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Observations on Antiviral Activity of Netropsin.* (20246)

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Many antibiotics and other substances which have been found to be active against viruses of the psittacosis-lymphogranuloma group also have antibacterial and/or anti-rickettsial activity. On the basis of such chemotherapeutic experiments Horsfall has pointed out(1) that perhaps the psittacosis-lymphogranuloma group should not be classified among the "true" viruses. The classification of the psittacosis-lymphogranuloma group as the family Chlamydozoaceae in the order Rickettsiales(2) supports this view. It is for treatment of infections with microorganisms of the order of Virales that active therapeutic substances are most conspicuously lacking(1,3-5). Some of the structural analogs of purine and pyrimidine constituents of nucleic acid have been reported to inhibit the multiplication of vaccinia virus in chick-embryo tissue culture(6), and some 5-phenoxypyrimidines, only moderately active inhibitors of vaccinia virus in chick embryonic tissue culture, have been shown to have a significant protective effect against intranasally or intracerebrally inoculated vaccinia virus in mice(7). One of the purine analogs, 2,6-diaminopurine, has been shown to increase the survival rate in mice infected with Russian Spring-Summer Encephalitis (RSSE) virus via the intraperitoneal route(8). This compound also inhibits RSSE virus in tissue culture(9). Two groups of investigators (10,11) have reported that several variously substituted thiosemicarbazones have *in vivo* antivaccinial activity. We have essentially confirmed these observations in the treatment of vaccinial infections in mice with substituted thiosemicarbazones(12). With the exception of the examples listed, chemotherapy of members of the order Virales has not been markedly successful(4).

In a search for effective antiviral agents being carried out in this laboratory we have tested numerous antibiotics and known tumor-inhibiting compounds for antiviral activity. Among the antibiotics tested was "Netropsin,"† isolated by Finlay *et al.*, from culture filtrates of *Streptomyces netropsis*(13). The

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* This work was carried out under a contract from the Chemical Corps Biological Laboratories, Camp Detrick, Frederick, Md.

TABLE I. Effect of Netropsin on Vaccinial Infection in Mice. Duration of treatment 5 days.

Treatment	Dosage (mg/kg)	Days of death							Survivor, total	% survivors	Challenge survivors, total
		5	6	7	8	9	10*	14			
N†	35				1	2		1	16/20	80	16/16
C			6	4	4	1			4/19	22	3/4
N	35				1				39/40	98	35/35
C			5	3		1	1		10/20	50	10/10
N	35				2		1		17/20	85	12/12
C			9	6	6	1			3/25	12	2/2
N	30						1		15/16	94	15/15
C		2	3	5	2	1			6/19	32	6/6
Totals N					4	2	1	1	88/96	92	78/78
C		2	23	18	12	4	1		23/83	28	21/22

* No deaths 11, 12 and 13th day.
† N = Netropsin; C = Controls.

present report deals with the effect of netropsin on vaccinial infections in mice.

Experimental. The virus used was the WR strain of mouse neurotropic vaccinia virus obtained from the American Type Culture Collection. The LD_{50} of this virus for intracerebrally inoculated mice is approximately 0.03 ml of a $10^{-4.5}$ dilution of virus infected mouse brain. Mice used were white Swiss, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. Mixed sexes weighing approximately 15 g apiece were employed in all experiments. Aqueous solutions of netropsin undergo change on storage, evidenced by changes in the chromatogram, ultraviolet absorption, antibiotic activity and toxicity(14). Mice were inoculated intracerebrally with one LD_{50} to one LD_{90} of virus and treatment was begun 30 to 60 minutes later. One-half a daily maximum tolerated dose (M.T.D.) of netropsin in aqueous solution was given intraperitoneally to each mouse morning and late afternoon for 5 days. Control mice were injected with distilled water at the same time the drug was given to the treated mice. Fourteen days after the first virus inoculation, all survivors were challenged intracerebrally with about 1000 LD_{50} of homologous virus, the challenge inoculation being given in the opposite cerebral hemisphere from the primary inoculation. The M.T.D. of netropsin for mice was taken as ⅓ the intraperitoneal LD_{50} dose. This was found to be 30-35 mg/kg/day for 5 days.

Table I summarizes a series of experiments in which mice infected with vaccinia virus via the intracerebral route were treated with netropsin.

It will be noted that netropsin markedly increased the survival rate of mice inoculated with vaccinia virus. It also prolonged the life span of those treated mice which died.

Mice which survived the initial inoculation of vaccinia virus almost invariably resisted challenge with homologous virus. The following experiment indicated that this immunity resulted from virus infection and not from the antigen present in the original inoculum. Mice were inoculated as in the experiments of Table I, but using a mouse brain suspension of virus which had been heated at 55°C for one hour on 2 occasions, 17 days apart. When challenged 14 days after the original inoculation with about 1000 LD_{50}, given intracerebrally, nearly all the mice died with typical signs of vaccinial infection. These observations essentially confirm previous reports that vaccinial immunity is not readily induced by inoculation with other than large amounts of inactive virus(15) and survivors of vaccinial infections resist challenge with multiple lethal doses of virus(16). These observations indicate that the increase in survival rates of netropsin-treated over control mice (Table I) is due to the activity of the drug in animals with active virus infections. Netropsin, administered at the M.T.D. for a period of days, results in marked weight loss in treated mice.

To determine whether the netropsin was protecting infected mice by some mechanism

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† Netropsin was supplied by Chas. Pfizer and Co., Brooklyn, N. Y.

TABLE II. Comparison of Starvation and Netropsin Treatment in Mice Infected with Vaccinia Virus.

Treatment	Days after inoculation with virus 0	2	6	8	Days of death 5	6	7	8	9	10	Survivor, total	% survivors	Resisted challenge, total
	Avg wt (g)												
Netropsin, 30 mg/kg/day, 5 days	14.9	13.7	11.8	11.9						1	18/19	95	15/15
Starvation	15.1	13.4	12.2	10.7	2	5	7	3	1		2/20	10	1/1
Controls	15.1	13.6	12.6	13.0	2	3	5	2	1		7/20	35	6/6

associated with weight loss the following experiment was carried out. Sixty mice were inoculated with vaccinia virus via the intracerebral route. They were then separated into 3 groups of 20 mice each. One group was treated with netropsin; a second group was left untreated and food was restricted but water was given *ad libitum;* and a third group was given control injections of water. All three groups were weighed at intervals and average weights were recorded. Results of this experiment, listed in Table II, indicate that weight loss similar to that seen in netropsin-treated mice does not account for the protective action attributed to the drug.

The effect of netropsin on intradermal vaccinial infections in rabbits was tested. Serial 10-fold dilutions of virus were inoculated intradermally into rabbits and M.T.D. amounts of netropsin were injected intraperitoneally into treated animals on a schedule of one-half daily dose twice a day for 5 days. No difference was noted in the skin infections between the control and drug-treated rabbits. It should be pointed out, however, that the M.T.D. of netropsin for rabbits is only about 20% of the M.T.D. for mice on a body weight basis, and therefore the rabbits did not receive as intensive drug treatment as did the mice.

The effect of netropsin on feline pneumonitis virus, influenza virus (Type A), Western equine encephalomyelitis virus (California strain), and poliomyelitis virus (Lansing strain) infections in mice was also tested and the drug showed no effect on any of these virus infections in mice.

We are at present testing the effect of this drug on variola virus infections. The details of these and other tests for antiviral activity of a number of other antibiotics and other materials will be published elsewhere.

Summary. Netropsin, an antibiotic from culture filtrates of *Streptomyces netropsis,* has marked therapeutic effect on experimental vaccinial infections (WR strain) in intracerebrally inoculated mice. It is ineffective against infections in mice with the viruses of feline pneumonitis, influenza, Western equine encephalomyelitis, and poliomyelitis.

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Effect of Emotional Stress, Anesthesia, and Death on Body Temperature of Mice Exposed to Cold. (20247)

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Hypothermia in small laboratory animals can be produced not only by exposure to cold(1) but can also be facilitated by wetting the body(2), lowering pO_2(3,4), binding the thorax(5), restraining(6), a strange environment(7,8), and binding the hind limbs together(9).

Although some maintain that exposure to conditions less severe than intolerable cold produces a fall in body temperature if the animal is also stressed, restrained or fearful (6-10), others indicate that body temperature does not fall in a restrained or stressed animal unless breathing is restricted(5). The data presented below seem to support the former, *i.e.*, a theory of "emotional hypothermia."

Methods and materials. Adult Swiss albino mice of both sexes were divided into 4 experimental groups: 18 unrestrained, 18 restrained, 18 anesthetized, and 6 dead animals, respectively. Seventy mg/kg of sodium pentobarbital was used for anesthesia and an overdose of the same anesthesia was used to produce death. The unrestrained mice were maintained individually in large cages which were placed in a refrigerator (6°C ± 2°) in which the restrained animals were simultaneously exposed in loose fitting cylindrical restraining cages. To minimize stress in the unrestrained animals, only initial and terminal (2 hr.) temperatures were taken, while in the other groups thermocouples inserted to a depth of 3-4 cm were left in the rectum and temperatures were taken intermittently throughout the exposure. Since others(5) have attributed the fall in body temperature, with similar restraining technics, to restricted breathing, another experiment was performed in which 48 mice were equally divided into controls and experimentals which were maintained for one hour each day in restraining cages at room temperature for a period of about 4 weeks. This "conditioning" was used to determine if mice could become accustomed to restraining cages so that the caging would fail to produce an emotional stress. At the end of this period of conditioning both groups were exposed in restraining cages to the cold (2°C ± 2°). One-half of these animals were exposed 3 days prior to the other half, the second group being used as controls on the first exposure. Initial and terminal (2 hr) temperatures were taken with a thermometer inserted rectally to a depth of 3 to 4 cm.

Results. The body temperatures of mice exposed in a refrigerator at a temperature of 6°C ± 2° are shown in Fig. 1. While there was little drop in body temperature of the unrestrained mice, body temperatures of the animals in the restraining cages fell to the point of death in approximately one hour despite continuous struggling. The rate of the temperature drop is compared in Fig. 1 with the body temperature drop of anesthetized and dead mice recorded under similar conditions.

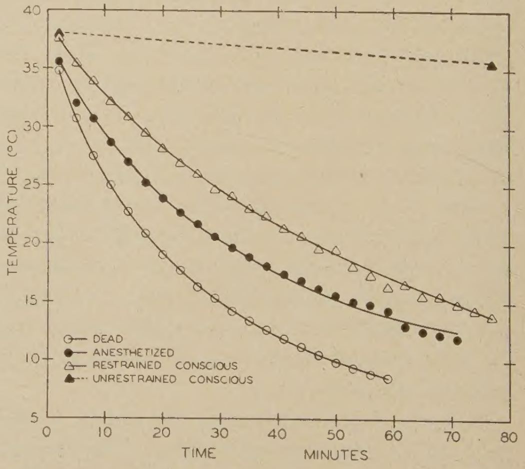

FIG. 1. Comparison of average temperature drop of dead mice, anesthetized mice, restrained conscious mice and unrestrained conscious mice in the cold (6°C ± 2°).

TABLE I. Effect of "Conditioning" on Body Temperature Drop of Mice in the Cold (2°C ± 2°).

Animal group	Initial temp., °C	Terminal temp., °C	% mortality
Controls— Not C* Not R*	38.3 ± 1.0	34.8 ± 1.6	0
R & not C	37.6 ± 1.2	13.0 ± 2.1	46
C & R	38.2 ± .9	19.3 ± 3.3	0

* C = conditioned; R = restrained.

In Table I is shown the comparison of the temperature drop in the conditioned and non-conditioned animals in a cold room (2°C ± 2°). As apparent from the figures, conditioning resulted in a lesser temperature drop in the animals in the restraining cages. Eleven of 24 of the non-conditioned animals died, while there was no mortality in the conditioned group.

Discussion. Although Ware, Hill, and Schultz(5) have reported that they were unable to produce hypothermia in rats without restricting breathing, there are several evidences of the existence of emotional hypothermia. Since in the present studies many of the mice were able to turn around in the restraining cages and in all cases a pencil could be moved between the back of the mouse and the cage, it was difficult to see how breathing could have been affected. Also, since the conditioned mice in our experiments were exposed to cold in an identical manner to the non-conditioned mice, it seems safe to conclude that the discrepancy of temperature drop between these two groups (Table I) cannot be attributed alone to the physical factors mentioned by Ware, but must also be attributed to emotional factors. It has also been observed in this laboratory that rats which live in restraining cages for a week or more do not lose body temperature on exposure to cold(10). The work of Phillips *et al.*(9) demonstrated that mice become hypothermic when the hind legs were bound together, a procedure which could not have restricted breathing appreciably.

It was also obvious that the body temperature drop did not result from restricted body movements, for the caged animals struggled until the lowering of body temperature resulted in slower movement and finally in no movement as the temperature neared the death point. The controls, which were caged individually, were quite motionless and usually huddled up with ruffled fur.

In their work with rats, Ware *et al.*(9). found that with tight taping of the chest a drop in body temperature could be produced which was faster than the drop in dead animals. This relationship was not observed in the present studies, for temperature drop in both anesthetized and dead mice was faster than the temperature drop of caged conscious mice.

Summary. Both restrained and unrestrained mice were exposed to cold. The unrestrained animals experienced a small drop in body temperature as compared to the restrained animals whose body temperatures dropped to the death point (approx. 10°C) in about one hour. Anesthetized and dead mice demonstrated greater temperature drops than were found in conscious restrained mice. Conditioning by maintaining the animals one hour per day in restraining cages resulted in a lesser temperature drop on exposure to cold as well as greatly decreased mortality. It was concluded that emotional hypothermia, not resulting from restricted breathing, can be produced in mice by the stress of confinement.

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Effect of Whole Body X-Irradiation on a Natural Inhibitor of Carboxypeptidase.* (20248)

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In the accompanying paper(1), we demonstrated the existence in many tissues of two carboxypeptidases and an inhibitor of one of these. The present paper describes experiments concerning the effect of whole body x-irradiation on these carboxypeptidase factors. It has been found that 400 roentgens significantly, and 600 roentgens totally, destroys the CP*a*I of the blood cells. Since it is probable that all tissue CP*a*I is actually contained in the blood cells present in the tissue at the time of sacrifice, it would be expected that whole tissue CP*a* activity would be increased by whole body x-irradiation. This has been found to be the case. It appears probable that the CP*a*I is contained in the white blood cells, although leukemic blood, like the blood of most other cancer-bearing animals or humans, is low in CP*a*I(2).

Experimental. Details of the assay procedure are given in the preceding paper(1), as are the method of preparation of the tissue for assay, and the definitions of units. Irradiation has been performed with a Keleket x-ray therapy machine operating at 250 KVP and 15 ma. One-half mm of Cu was used as filter. X-ray dose was determined with a Victoreen r-meter, the thimble being placed for calibration in a position corresponding approximately to the mid-section of the animal. The dose rate was generally 30-40 r per minute.

In preliminary studies, the effect of 650 r of whole body x-radiation was determined on 3 different catheptic enzymes in liver, kidney, and intestine of mice. Enzymes tested were the Cathepsins II, III, and IV of Fruton *et al.* (3). For these, the substrates used were, respectively, benzoylargininamide, dl-leucylglycine, and chloracetyltyrosine. Table I indicates that the Cathepsin II of liver, kidney, and intestine, the Cathepsin III of intestine, and the Cathepsin IV (carboxypeptidase) of kidney are all increased by this treatment. The overall catheptic reaction, as measured by Anson's(4) hemoglobin digestion method, was not affected in any tissue.

Whole body x-irradiation might be considered to increase whole kidney carboxypeptidase in either or both of two ways: The enzyme might be increased in activity, or an inhibitor might be destroyed. Other possibilities, such as formation of an activator, seem less likely. The situation is further complicated by the possibility that the x-ray effects are not on the kidney tissue itself, but on the blood present in the kidneys at the time of sacrifice.

These possibilities were simultaneously analyzed by the following set of experiments: a) Kidney perfusion. In each of 4 rats under ether anesthesia, one kidney was thoroughly perfused with 0.9% saline in order to wash out all blood. The enzyme fraction (centrifuged supernatant) was totally unaffected by the perfusion, while the inhibitor activity (centrifuged precipitate) was reduced by an average of 93%. b) Kidney shielding. Four rats were x-rayed (650 r) while firmly restrained within a lead belt which covered the kidney region and effectively reduced the x-ray intensity in air from 35 r/minute outside the shield to 0.5 r/minute within. At the same time, 4 rats were irradiated without being shielded. Twenty-four hours after x-ray, these 2 groups were sacrificed, as were four non-irradiated rats which, like the x-rayed animals, had been starved (water *ad lib.*) for the 24 hours. The kidneys were removed, homogenized, and centrifuged, and the fractions were assayed for CP*a* and CP*a*I. Table II indicates that the enzyme was not affected,

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† The present work was submitted by John C. Ballin in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology.

TABLE I. Effect of 650 r of Whole Body X-Radiation on Proteolytic Activities of Mouse Tissues.

	Liver			Kidney			Intestine		
	Control	X-rayed	Student's t	Control	X-rayed	Student's t	Control	X-rayed	Student's t
Cathepsin II	.2 ± .2	1.1 ± .5	4.8	.4 ± .3	1.2 ± .5	4.1	.8 ± .4	2.8 ± 1.0	5.1
III	.0	.0	.0	1.0 ± .4	1.4 ± .5	1.9	.9 ± .3	1.7 ± .4	4.2
IV	.0	.0	.0	1.9 ± .6	4.4 ± 1.0	6.0	.0	.0	.0
Hemoglobin digestion	1.59 ± .46	1.57 ± .48	.06	1.10 ± .35	1.11 ± .35	.04	1.42 ± .55	1.55 ± .46	.39

For Cathepsins II, III, and IV, values are expressed as milliequivalents increase in alkali titration; for hemoglobin digestion, values are expressed as mols tyrosine solubilized. All are on the basis: per mg protein. Data are mean and average deviation from the mean. Population is 10 mice for each point in the cathepsin series, 7 mice for each point in hemoglobin digestion.

TABLE II. Effect of Kidney Shielding on Carboxypeptidase and Carboxypeptidase Inhibitor of Kidneys of Rats Sacrificed 24 Hours after 650 r X-rays.

Irradiated	Supernatant (enzyme)	Precipitate (inhibitor)
Not (control)	22.7 ± 2.0	20.0 ± 5.1
Whole body	21.4 ± 1.3	13.0 ± 1.1
Whole body, kidney shielded	24.9 ± 2.3	4.1 ± 1.7

Data presented are mean and avg dev. from the mean. Each figure represents avg of 4 rats, expressed as carboxypeptidase units, or carboxypeptidase inhibitor units, per ml of 10% kidney homogenate.

while the inhibitor was reduced by the irradiation. c) Blood cell inhibitor assay. Fig. 1 shows the effect of 650 r of whole body x-irradiation on the blood cell CP*a*I of rabbits. It will be observed that the CP*a*I drops precipitously, reaching a minimum value at 2 days and returning essentially to normal by the seventh day. The decrease in the CP*a*I in the blood of non-irradiated rabbits is probably due to the 48-hour starvation period imposed upon both sets of animals.

The data from these experiments would seem to necessitate the following conclusions: 1) The only CP*a*I found in kidney is that due to the kidney's content of blood at the time of sacrifice; 2) The CP*a* of kidney is not affected by x-irradiation with the doses employed; and 3) the CP*a*I of blood cells is destroyed by whole body x-irradiation.

A crucial experiment to assure the validity of these conclusions would be the whole body x-irradiation of animals, with subsequent assay of the inhibitor of the kidney. If all kidney inhibitor is actually blood inhibitor,

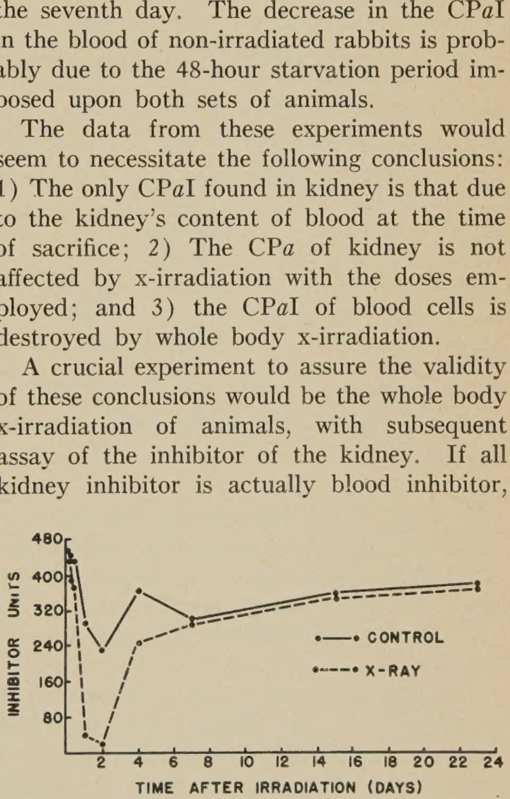

FIG. 1. Effect of 650 r whole body x-irradiation on rabbit blood cell carboxypeptidase inhibitor (CP*a*I). Each datum represents avg of five animals. Inhibitor activity is expressed as units per ml packed blood cells.

TABLE III. Effect of 650 r Whole Body X-Irradiation on Carboxypeptidase *a* (CP*a*) and Carboxypeptidase *a* Inhibitor (CP*a*I) of Rat Kidney.

Rats used			Treatment	Component assayed	
Strain	Sex	Wt, g		CP*a*	CP*a*I
Maguran	♂	250-300	C*	17.8 ± 1.6	11.1 ± .4
			X	20.3 ± 1.8	10.0 ± 1.4
Sprague-Dawley	♀	150-200	C	18.2 ± 3.5	12.6 ± 2.4
			X	13.4 ± .8	3.6 ± .4
” ”	♂	250-300	C	16.8 ± .8	12.5 ± .4
			X	16.1 ± .8	2.9 ± 1.5
” ”	♀	” - ”	C	20.7 ± 1.8	15.0 ± .4
			X	18.9 ± 2.3	3.7 ± 1.3
” ”	♂	150-200	C	17.1 ± .7	12.9 ± .5
			X	16.9 ± 1.8	4.1 ± 1.5
Maguran	♂	250-300	C	17.6 ± 1.1	14.0 ± 1.1
			X	17.1 ± 1.1	3.5 ± .5
”	♂	” - ”	C†	22.7 ± 2.0	20.0 ± 5.1
			X†	21.4 ± 1.3	13.0 ± 1.1

Data are mean and avg deviation from the mean, expressed as enzyme or inhibitor units per ml 10% kidney homogenate. Each figure represents the avg of 5 or 6 rats in the first experiment and of 4 rats in each succeeding one. Rats sacrificed 24 hr after irradiation.

* C = control; X = x-rayed.

† Data repeated from Table II.

TABLE IV. Effect of Whole Body X-Radiation at Various Dose Levels on Blood Cell Carboxypeptidase Inhibitor of Rabbits, Guinea Pigs, and Rats 24 Hours Later.

Species	Approx. LD_{50} of 250 KVP X-rays (roentgens)	X-ray dose, roentgens							
		0	175	200	225	400	600	650	800
Man	400 (est.)*	656±36 (19)							
Rat	650	184±18 (23)		186±15 (3)		126±30 (3)	2±3 (3)		1± 2 (3)
Guinea pig	225	350±16 (4)	384±20 (4)		378±24 (4)			46±22 (3)	
Rabbit	790	310±66 (45)				256±13 (4)			26±20 (4)

Data given are mean and avg deviation from the mean, expressed as inhibitor units per ml packed blood cells. Figures in parentheses indicate number of individuals.

* Ref. 6.

and if all blood inhibitor is destroyed by whole-body x-irradiation, then it follows that whole body x-irradiation should destroy kidney inhibitor. The data are presented in Table III, which indicates this conclusion to be valid.

The effect of whole body x-irradiation in reducing the blood cell CP*a*I is of particular interest as a possible biological "dosimeter," a means of measuring the dose of ionizing radiation received by an individual human or animal. Since such dosimeter may measure dose either as a function of dose lethality or as a measure of absolute amount of radiation received, several animal species of widely varying sensitivity to x-rays have been tested at a variety of doses. The data of Table IV indicate decisively that the blood cell CP*a*I test measures absolute amounts of radiation received, rather than measuring the percentage of lethal dose.

Evidence indicates the white blood cell to be the source of the inhibitor. In one experiment, we repeatedly washed, by centrifugation, a tube of packed rabbit blood cells, trying with each washing to suck off as much as possible of the "buffy layer" of white cells. After three washings, the packed cells gave a negligible inhibitor activity, while the washings, containing the buffy layer, showed inhibitor activity. In another experiment, packed cells were laked with 20 volumes of water, then centrifuged. A slight precipitate, presumably rich in the debris of difficultly lysed white cells, appeared. When this precipitate was resuspended and assayed for inhibitor, the bulk of the initial inhibitor activity was found in this fraction; the supernatant was devoid of activity. That the inhibitor is not merely washed off or out of the red cells is indicated by the fact that our routine assay of inhibitor involves repeated washings of the red cells with saline, care being taken, however, not to remove the buffy layer. It is therefore concluded that the white cells are the source of the carboxypeptidase inhibitor.

Discussion. Although as yet not decisively proven, it appears probable that the source of the inhibitor is the white blood cell. The relatively great activity of the inhibitor as compared to most tissue enzymes would argue for the source being the erythrocyte, but the experiments cited above indicate otherwise. In addition, some theoretical considerations favor the white cell as the source. We have shown that whole body x-irradiation reduces the blood cell inhibitor greatly by the second day, with return to normalcy at about a week. This would correspond to the life span of, say, a polymorphonuclear leukocyte, but is much too fast an action to be explained on the basis of an effect on newly formed red cells. Jacobson and co-workers(5) have shown that after LD_{50} doses of whole body x-rays, a lymphopenia appears in a few hours, a granulocytopenia in less than a week, and red cell effects only after 4-6 weeks. It is possible, of course, that the actual source of the inhibitor is the platelet or some plasma-insoluble substance.

Although the reduction by whole body x-irradiation of the blood cell CP*a*I does not measure the administered radiation as a per cent of the lethal dose, it still seems to us to be of great interest and potential value in estimating the lethality of a dose received by a human being. In this connection, it is a fortunate circumstance that the dose range measured by this test is one of practical interest in the study of man. If we assume an LD_{50} for man of 400 r(6), and further assume that the test applies to man in a fashion similar to the rodents tested, then it follows that a determination of the blood cell carboxypeptidase inhibitor will indicate at least whether or not the individual has received a lethal dose of radiation.

It is improbable that the x-ray effect on blood cell CP*a*I is directly related to the lethal effects of the radiation. This conclusion is drawn from two facts: First, the reduction by whole body x-irradiation of blood inhibitor is dependent on the absolute amount of radiation received, not on the degree of lethality in the particular species. For example, it is possible to irradiate guinea pigs at lethal levels without affecting the blood cell CP*a*I. Second, we have been unable to detect any CP*a*I whatsoever in the shed blood of mice or dogs. This is of particular interest because, in the mouse at least, whole body x-irradiation does increase the whole kidney carboxypeptidase activity. The difference between this mechanism in the rat and the mouse has not yet been elucidated.

Summary. Whole body x-irradiation of mice, rats, and rabbits at the LD_{50} range increases the activity of certain tissue cathepsins. Liver, kidney, and intestine were the tissues tested, and it was found that one of 3 liver cathepsins, 2 of the same 3 cathepsins in kidney, and 2 of the 3 in intestine were increased in activity by the radiation. This appears due, at least in the case of rat kidney carboxypeptidase, to the destruction by the x-rays of an enzyme inhibitor. It is possible that the source of all inhibitor found in tissues and blood alike is the blood cell, probably the leukocyte. The possibility is suggested that the reduction of carboxypeptidase inhibitor in blood cells may serve as a basis for determining, within 24 hours of the exposure, whether or not an individual has received a lethal dose of ionizing radiation.

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Carboxypeptidase in Mammalian Tissue.* (20249)

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The term "carboxypeptidase" is used in two distinctly different senses. On the one hand, it refers specifically to the enzyme entity first isolated in crystalline form from pancreas by Anson(1). On the other hand, it is used in more general reference to an enzyme or group of enzymes found in any of several tissues and classified by Fruton, Irving, and Bergmann(2) as Cathepsin IV, and later by Tallan, Jones, and Fruton(3) as carboxypeptidase. The pancreatic carboxypeptidase is usually assayed at pH 7.3-7.8 in the absence of cysteine, which Smith and Hanson(4) found actually to inhibit this enzyme. The carboxypeptidase (Cathepsin IV) of spleen and kidney was measured by Fruton, Irving, and Bergmann(2) at pH 5.0-5.1, and 0.01 M cysteine was used for maximum activity.

Work in this laboratory has demonstrated the existence in a wide variety of tissues of both types of carboxypeptidase. We have designated as carboxypeptidase *a* (CP*a*) that enzyme which requires cysteine for activity and which has optimal activity at pH 5.5-6.5 and 8.0-8.5. This enzyme has not previously been detected in many tissues because, first, it is much less active in the neighborhood of pH 7.5, and second, because most tissues also contain a cellular inhibitor of this enzyme. We have designated as carboxypeptidase *b* (CP*b*) that enzyme which has pH optima of 5.5 and 7.5 and is active in the absence of cysteine; in fact, the cysteine concentration used in the assay of CP*a* completely inhibits CP*b*. The natural tissue inhibitor of CP*a* is without effect on CP*b*. The inhibitor is apparently protein in nature, is generally found with the particulate matter in centrifuged homogenates, and inhibits the enzyme in a non-competitive fashion.

Experimental. Animals used were normal, Sprague-Dawley or Maguran rats and Carworth Farm mice. In general, CP assays were carried out by incubating a given tissue fraction with chloracetyl-L-tyrosine, with or without cysteine, for 10 minutes at 37°C. The chloracetyl-L-tyrosine was present in a final concentration of 0.0088 M. Tissue preparation, substrate, and cysteine were each brought to pH 5.5 before the assay. Aliquots were removed immediately after mixing, and again exactly ten minutes later, into 10 volumes of absolute alcohol. The 2 alcoholic solutions were then titrated with alcoholic KOH, approximately 0.01 N, using thymolphthalein as indicator. The titration thus resembles that of Grassmann and Heyde(5). That the titration increment is directly proportional to the amount of enzyme source added is demonstrated by the data of Fig. 1. Protein was determined by the method of Robinson and Hogden(6), as modified by Keyser and Vaughn(7).

Results. Efforts were first directed at the detection of CP in a variety of tissues. When whole homogenates (9 ml H_2O per g wet tissue) were used with 0.0088 M cysteine present, enzyme activity was detected in rat kidney but not in liver or intestine. The

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† The present work was submitted by John C. Ballin in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology.

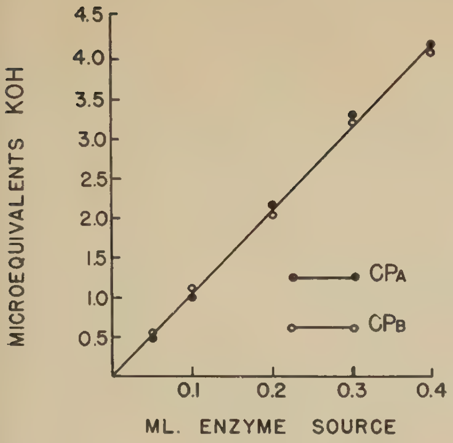

FIG. 1. Linearity of titration increment with respect to amount of enzyme source. Ordinate represents difference in titration of aliquots taken immediately after mixing, and 10 min. later. Abscissa gives volume added of approximately 40% rat kidney homogenate. The unusually concentrated enzyme source was used in order to permit a wider variety of aliquot sizes. pH = 5.5; temp. = 37.0°C.

possibility was investigated that some "inactive" tissues might actually contain CP, and also a CP inhibitor (CPI), which would prevent the demonstration of CP activity. This consideration was encouraged by the finding that the CP activity of whole rat kidney homogenate was increased 23% by shaking with $CHCl_3$, with a simultaneous reduction in protein content of 53%. There was thus obtained an increase of 180% in CP activity per mg protein.

If the $CHCl_3$ phase is evaporated to dryness at room temperature and the residue resuspended in water, it has no effect on the CP activity of the aqueous phase; it thus appears likely that treatment with $CHCl_3$ destroys the inhibitor, probably by denaturation.

In order to obtain the inhibitor in assayable form, an attempt was made to separate it by differential centrifugation after water homogenization.

In early experiments, 4 fractions were separated, representing cellular debris and nuclei (700 g), mitochondria (5,000 g), a high-speed fraction (18,000 g), and the residual supernatant. The bulk of the CP activity is found in the high-speed supernatant. If we arbitrarily assign the value of 100 CPU/mg protein to this fraction, then the activities of the other fractions of a rat kidney homogenate in a typical experiment were: whole homogenate, 13; nuclear fraction, 0; mitochondria, 15; and high-speed precipitate, 0. Because of this distribution, our routine technic has been to homogenize the tissue with nine volumes of cold distilled water, then centrifuge for 30 minutes at 18,000 g in the cold. The supernatant is assayed for CP activity, and the precipitate is re-suspended by homogenization with the original volume of cold distilled water and assayed for inhibitor.

It has been found that the amount of inhibition is linearly proportional to the volume of well-suspended inhibitor source added, and so we have defined our enzyme and inhibitor units as follows: a) Carboxypeptidase unit (CPU): that amount of enzyme which causes

TABLE I. Distribution of Carboxypeptidase *a* and *b* and Carboxypeptidase *a* and *b* Inhibitor in Normal Rat Tissue.

Tissue	Whole homogenate	Supernatant (enzyme fraction)	Precipitate (inhibitor fraction)
	CP*a*U	CP*a*U	CP*a*IU
Blood	—	4	130
Kidney	60	132	72
Spleen	0	59	89
Liver	3	59	59
Testis	17	59	104
Intestine	4	93	97
Heart	0	131	118
Brain	63	143	101
Lung	0	56	46
Stomach	66	69	0
Skeletal muscle	0	6	16
	CP*b*U	CP*b*U	CP*b*IU
Blood	—	3	0
Kidney	162	171	3
Spleen	85	56	3
Liver	74	59	0
Testis	8	59	0
Intestine	84	84	13
Heart	80	72	0
Brain	80	76	0
Lung	6	16	7
Stomach	56	49	3
Skeletal muscle	3	0	0

Blood divided into cells (here "precipitate") and plasma (here "supernatant") by low speed centrifugation. Other tissues homogenized in H_2O, and a portion centrifuged at 18000 g for 30 min. Whole homogenate and supernatant assayed for carboxypeptidase *a* (CP*a*) and carboxypeptidase *b* (CP*b*); precipitate returned to original volume with water and assayed for CP*a* inhibitor (CP*a*I) and CP*b* inhibitor (CP*b*I). All figures based on 1 g (or in case of blood, 1 ml) original wet tissue.

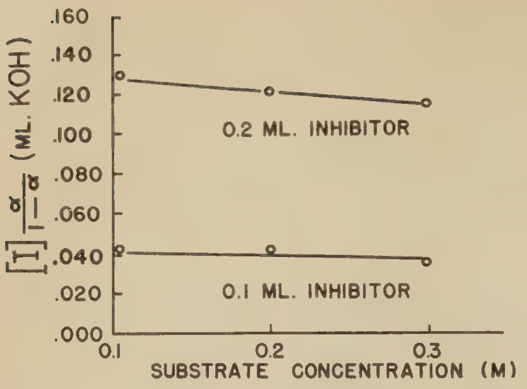

FIG. 2. Non-competitive nature of carboxypeptidase inhibition by rat kidney residue fraction. I = conc. of inhibitor; V and V_1 = velocity in absence or in presence of inhibitor, respectively; $\alpha = \frac{V_1}{V}$.

an increase of one microequivalent of alkali titration under our standardized conditions, and b) Carboxypeptidase inhibitor unit (CPIU): that amount of inhibitor which reduces the enzyme activity of any CP preparation by one CPU.

By the use of this centrifugation technic, we have found that CP is more widely distributed than generally recognized, but its presence is masked in whole tissue preparations by the simultaneous presence of inhibitor. Table I indicates the extent of this distribution. In Table I, 20 units of enzyme or inhibitor is considered the approximate limit of accuracy of the method. This high value is due to a large dilution factor. The actual titration is reproducible to approximately 0.1 microequivalent of alkali.

That the inhibitor is non-competitive in its action is shown by Fig. 2, the results of an experiment based on the suggestion of Massart(8) for determining the type of inhibition. That it is probably protein in nature is indicated by the data of Table II where it is seen that the inhibitor is heat-labile and non-dialyzable. The non-dialyzability, however, may mean only that the inhibitor is in suspension and not in true solution. Because of the possibility that the blood content of the various tissues may be responsible for all inhibitor noted, hemoglobin was tested for inhibitor activity and found negative. This question is further discussed below. Urine also was found to be inhibitor-free.

All of the above results were obtained with experimental systems containing 0.0088 M cysteine at pH 5.5. The possibility was next considered that two different enzymes may be present. This has proven to be the case.

Until such time as 2 distinct enzymes are actually isolated, the demonstration that 2 separate entities are involved, and that no overlapping of activities occurs, must be an indirect one. Data of a typical experiment are presented in Table III. These data, it seems to us, are very suggestive of one particular set of interpretations. Since the inhibitor fraction inhibits all CP*a* activity (*i.e.*, in presence of cysteine), but has ***no inhibitory effect whatsoever in the absence of cysteine,*** then no part of the CP*b* activity (*i.e.* in absence of cysteine) can be attributed to the CP*a*. On the other hand, since it is seen that the cellular inhibitor has no effect on CP*b*, yet permits ***no residual activity whatsoever in the presence of cysteine,*** then no part of the CP*a*

TABLE II. Evidence for the Protein Nature of Carboxypeptidase Inhibitor.

Addition to standard enzyme	CP*a*U/ml	CP*a*IU/ml
None	32	—
I,* freshly prepared	20	12
I, heated 1 hr at 37°C	20	12
I, " 3 min. at 100°C	32	0
None	28	—
I, 24 hr in cold	17	11
I, dialyzed 24 hr in cold	18	10

* I = Inhibitor fraction.

Normal rat kidney homogenized in H_2O and centrifuged at 18000 g 30 min. Supernatant assayed for carboxypeptidase *a* (CP*a*); precipitate returned to original volume with H_2O and assayed for carboxypeptidase *a* inhibitor (CP*a*I). All units based on 1 g original wet tissue.

TABLE III. Evidence for Co-Existence of Two Carboxypeptidases.

Components present	Titration increase, meq.
Enzyme + cysteine	.54
" + cysteine + inhibitor	.00
" alone	.30
" + inhibitor	.31

"Enzyme" and "inhibitor" were supernatant and precipitate fractions, respectively, of homogenized, centrifuged rat kidney. Cysteine was present, where indicated, at 0.0088 M.

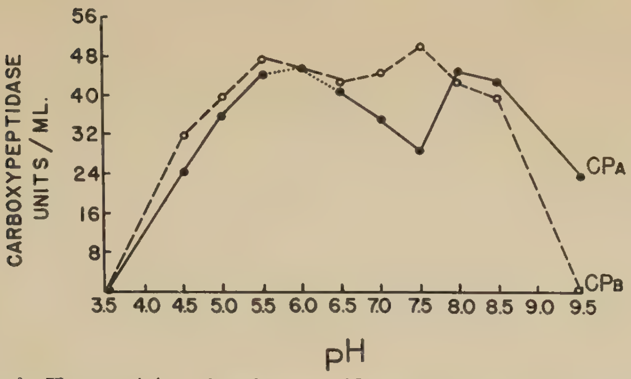

FIG. 3. Effect of pH on activity of carboxypeptidase *a* and *b*. Technical difficulties made it necessary to determine the pH 6 point in a separate experiment, at which time the pH 5.5 and 6.5 points were repeated for purposes of curve-fitting. This is the reason for the dotted area of the curve.

activity can be attributed to the CP*b*. This question is further discussed below.

Fig. 3 shows pH activity curves for CP*a* and CP*b* in freshly prepared kidney homogenate supernatant. It will be noted that pH optima for CP*a* are at pH 5.5-6.5 and 8.0-8.5, and for CP*b*, pH 5.5 and 7.5. It is of particular interest that at pH 7.5, where the cysteine-inhibited carboxypeptidase is ordinarily assayed, the cysteine-requiring enzyme has minimal activity. In some preparations we have actually failed to detect any CP*a* activity whatsoever at pH 7.5, while considerable activity is shown at pH 5.5. From the fact that each enzyme apparently has two pH optima, the possibility must be considered that we actually are dealing not with 2, but with 4, enzymes. However, no other evidence has been observed to bolster this possibility.

Discussion. The possibility that all observed inhibitor of tissues may be due to the blood cells present in the tissue is further discussed in the succeeding paper. This possibility is fortified by the data of Table I, in which the relatively enormous concentration of inhibitor in blood cells is apparent. The possibility has been suggested that the entire phenomenon of inhibition may be merely one of adsorption of the enzyme onto particulate matter, since the inhibitor has not yet been separated from a particulate fraction. This seems to us unlikely, for two reasons: First, if it were so, the amount of inhibition should be critically dependent upon the fineness of re-suspension of the precipitate fraction; we have never detected such dependence. Second, if this were so, it seems unlikely that we could ever separate enzyme and inhibitor by simple centrifugation. The critical question yet remains, however, as to just how the inhibitor does function, since we have thus far failed to obtain the inhibitor in true solution.

The evidence given in Table III for the co-existence of two carboxypeptidases cannot be considered absolute proof. Other, though less likely, possibilities may be suggested. For example, it is conceivable that the cysteine activates not only carboxypeptidase *a*, but the inhibitor as well; if this is so, then the line of reasoning presented is flawed.

The possibility has been considered that the enzyme we have chosen to call carboxypeptidase *b* is actually identical with the cathepsin I of Fruton *et al.*(2). Definitive proof is difficult to obtain, yet one piece of evidence appears to favor the non-identity of CP*b* with cathepsin I. Fruton *et al.* refer to the thermolability of cathepsin I as a distinguishing feature; their data show a 52% decrease in activity of cathepsin I in beef kidney, and a 24% decrease in swine kidney, after heating for 15 minutes at 50°C. Under these circumstances, we have found the CP*b* of rat kidney to be decreased only 8%, while the CP*a* in the same preparation was decreased 26%. Although suggestive this cannot be considered firm proof, however, because of the possibility of species variation in this respect.

The possibility was also considered that our

CP*a* and CP*b* may be, respectively, the pro-carboxypeptidase and carboxypeptidase of Anson(9). To test this, a portion of kidney supernatant was treated with trypsin according to Anson's method for assay of the enzyme and zymogen. The treated and untreated portions were then assayed for CP*a* and CP*b*. The only effect of the trypsin treatment was an overall diminution in both enzymes; the ratio of the two was unaffected. It is thus apparent that CP*a* is not a trypsin-activatable precursor of CP*b* and so is not identical with Anson's pro-carboxypeptidase.

Preliminary experiments indicate that if a kidney homogenate is permitted to stand at room temperature for several days under a layer of toluene, the amount of CP*b* increases at the expense of CP*a*, which decreases to an approximately equivalent degree. This interconversion is apparently enzymatic in nature and occurs only at certain pHs, of which 8.5 appears to be optimum. The reverse reaction has not been detected.

Summary. Evidence is presented that the enzyme carboxypeptidase is more widely distributed throughout mammalian tissues than is commonly realized. The reasons for previous failure to detect the enzyme are: a) the existence of a natural cellular inhibitor, the effect of which is to mask the enzymatic activity, and b) the fact that two carboxypeptidases probably co-exist in many tissues; the pH optima of these two are different, and one is activated, the other inhibited, by cysteine. The natural cellular inhibitor affects only one of the carboxypeptidases, and does so in a non-competitive fashion.

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Development of Sarcomas in Marsh-Albino Mice Following Injection of Desoxycorticosterone Acetate in Sesame Oil.* (20250)

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While investigating strain differences in mice, an incidental observation was made in the Marsh strain, namely the development of tumors following prolonged injections of a mineral corticoid, desoxycorticosterone acetate (DCA; Organon's Doca Acetate) in sesame oil. This development has interest in view of the numerous reports that the administration of DCA in various forms has no tumorigenic effect [Kuhlman *et al.*(1), Carnes *et al.*(2), Ludden *et al.*(3), Shimkin and Grady(4), Shimkin and White(5), Burrill and Greene(6), Forbes(7), Rakoff *et al.*(8), Rodard and Freed(9), Gineste *et al.*(10), Sarason(11), Selye and Hall(12), Selye *et al.*(13), Bruzzone *et al.*(14), Segaloff(15), and Lipschutz(16)]. Shimkin and Wyman(17), however, implanted DCA pellets in C3H mice, and mammary tumors developed. These authors considered the results to be insignificant in view of the small number of animals involved; furthermore, the C3H strain is known to produce spontaneous mammary adenocarcinomas at an early age. Woolley

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* We wish to express our sincere appreciation to Dr. Kenneth Wade Thompson of Organon Inc. for the generous donation of desoxycorticosterone acetate (Doca) and to Dr. Robert Gaunt for his kind assistance.

TABLE I. Summary of Experimental Procedure on Marsh-Albino Mice. 12 animals in each series.

Sex	Treatment	Duration of treatment, mo	Animals surviving 3 mo after termination of treatment No.	%	Incidence of tumor development 6 mo after termination of treatment No.	%
♀	None	—	12	100	0	0
♀	Doca acetate, .2 mg	3	11*	91	10†	91
♂	None	—	12	100	0	0
♂	Doca acetate, .2 mg	3	9*	75	9	100

* Four died during treatment; cause of death unknown.

† One killed 3 mo after termination of treatment; no tumor evident.

and Chute(18) concluded that DCA, provided in pellet form, does not restrict the development of adrenal tumors. The work of Houssay *et al.*(19) indicates that DCA restricts adrenal carcinoma development.

Procedure. Six-week-old female and male Marsh-albino mice, weighing 20-25 g, were used. All animals were fed Purina Laboratory Chow and water *ad lib.* DCA in sesame oil (0.2 mg/0.1 cc) was given subcutaneously daily except Saturdays and Sundays for 3 months. The injections were made alternately into the left and right flanks. After the 3-month period of injections, the animals were examined periodically. At death the animals were autopsied; the tissues, fixed in formalin and stained with Hematoxylin and Eosin, were then studied histologically.

Results. The results of this experiment are shown in Table I. Of the 24 mice injected, 4 died within 3 weeks of the initiation of the experiment. Toward the end of the period of inoculation and for several weeks thereafter, all the mice showed fairly large subcutaneous swellings on the dorsal and lateral surfaces; these fluctuated on palpation. The swelling in one animal sacrificed during this period was found to be an oil-filled cyst the wall of which, upon microscopic examination, was found to be devoid of tumor. The remaining mice were kept under observation for several months.

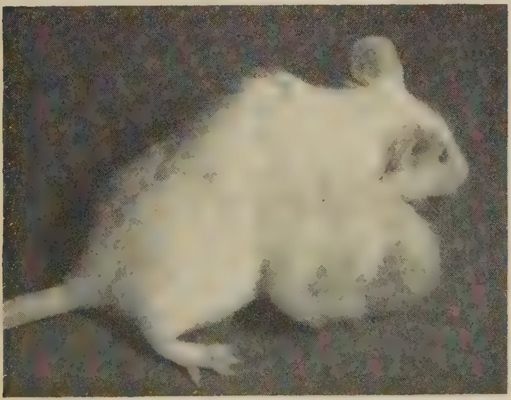

FIG. 1. Tumor masses evident on dorsal and lateral regions of a Marsh mouse. The dorsal mass was diagnosed as a fibrosarcoma and the lateral mass as a myosarcoma.

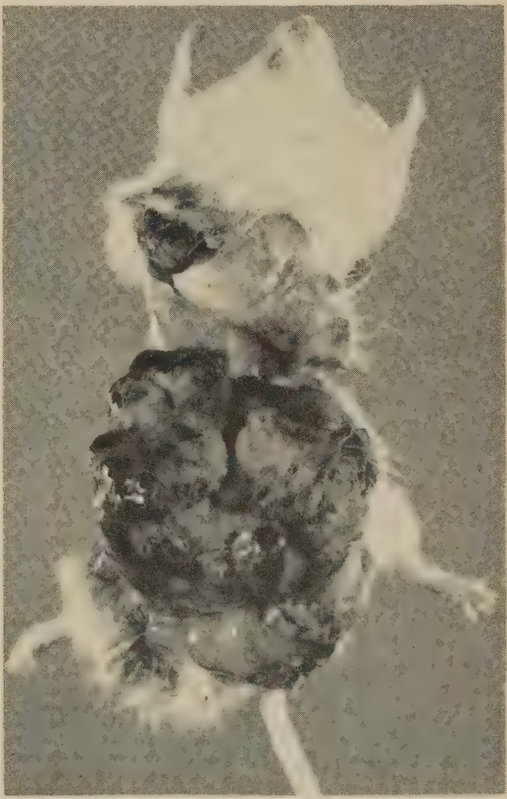

FIG. 2. Tumor mass evident on the dorsal region of a Marsh mouse. This tumor mass comprised 48% of total body wt of the animal.

TABLE II. Transplantation of DCA-Induced Sarcomas in Marsh-Albino Mice.

Tumor No.	Tumor	No. of transplantations ♂	♀	No. of takes* (F_1 generation)	% takes (F_1 generation)
1	Fibrosarcoma	15	4	19	100
2	”	10		10	100
3	”	5	5	10	100
4	Myosarcoma	5	15	20	100
5	”	10		8	80
6	Fibrosarcoma	10	10	20	100
7	”	12	8	20	100
8	Myosarcoma	4	4	8	100
9	Fibrosarcoma	9	1	10	100

* Showing progressively enlarging tumors.

During this period the oil cyst persisted, becoming smaller but gradually harder, until they presented a hard, firm mass with no evidence of a liquid content on palpation. Approximately 6 months after the cessation of inoculations (9 months after the initiation of the experiment), all of the mice showed large, firm masses as shown in Fig. 1 and Fig. 2. In some cases the induced tumor comprised as much as 50% of the total body weight of the animal.

Nine of the mice having the largest masses were sacrificed and, upon autopsy, showed well-circumscribed subcutaneous firm masses which were adherent to the overlying skin. The masses, when cut, were pearl gray in color; in some, small oil pockets were found in the center. These masses were examined microscopically. All were diagnosed as malignant tumors: 3 as myosarcoma and 6 as fibrosarcoma. In many instances the cells of these tumors were arranged in interlacing bundles, while other tumors or other regions of the same tumor contained cells that were round or irregular in shape. Mitoses and giant multinucleated cells were evident throughout most of the tumors. In some, the blood channels were prominent and indicative of a hemangiomatous structure. The tumors were encapsulated, and there was no evidence of metastasis in the lungs, heart, liver, spleen, kidneys, adrenals, reproductive organs, etc.; however, the liver and kidney in some animals showed definite evidence of hypertrophy. The ovaries showed evidence of stimulation.

Fragments of the tumors from 9 animals were transplanted into mice of the same strain. To date, all of the transplants except two are growing. (Table II).

The remaining 10 mice all have firm tumors up to 4 cm in diameter. They are being watched for signs of metastasis.

Because of the original intent of this experiment, there were no control animals which received inoculations of sesame oil only. However, in other experiments, mice of the Marsh strain have received sesame-oil injections for a shorter period of time without showing any evidence of tumor formation.

Summary. Twenty-four mice of the Marsh-Albino strain were injected daily for a period of 3 months with desoxycorticosterone acetate (0.2 mg/0.1 cc) in sesame oil. Of these, 1 was killed 3 months after termination of treatment but showed no evidence of tumor, while 9 others, killed 6 months after termination of treatments had subcutaneous tumors of two histological types, fibrosarcoma and myosarcoma. The remaining 10 mice are still alive; all show gross evidence of tumor formation, which became evident 6 months after completion of treatment.

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Effect of Orally Administered Penicillin-Resistant Microorganisms on Growth of Chicks.*† (20251)

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(Introduced by G. M. Briggs.)

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The exact mechanism of antibiotic stimulation of chick growth is still not clearly understood. Most theories concerning this mechanism explain it through changes in the intestinal microflora. These have been reviewed by Briggs(1). In experiments by Romoser *et al.*(2), definite changes occurred in the number and types of bacteria found in the ceca of four-week-old chicks following the oral administration of procaine penicillin G. The numbers of *Escherichia coli* and *Aerobacter aerogenes* present in the ceca were increased when penicillin G was fed at a level of 150 ppm of the diet. The growth rate was also improved. The addition of 5-15% lactose, in combination with the antibiotic, further increased chick growth and the numbers of these organisms. Anderson *et al.*(3) observed an increase in the coliform bacteria as a result of feeding penicillin to chicks. Similar increases, though less marked, were also secured by March and Biely(4) who used aureomycin. These workers also noted a marked decrease in the lactobacilli. Preliminary reports indicate that oral administration of *A. aerogenes*(5) or *E. coli*(6) increase the growth rate of chicks fed diets containing penicillin. From this it would appear that the growth-promoting action of antibiotics may result from: 1) the elimination of fastidious organisms which compete with the host for essential known or unidentified nutrients and/or, 2) the stimulation of other organisms which affect the host beneficially through the *in vivo* synthesis of one or more growth factors.

In this study the intestinal microflora was modified by feeding viable penicillin-resistant organisms to chicks, in order to investigate further the effect on chick growth.

Materials and methods. In all experiments, 15-20 day-old New Hampshire chicks of both sexes were used per experimental group. In Exp. 1, the chicks used were the progeny of dams housed on raised wire floors and fed a ration which was complete in all known nutrients but contained no animal protein supplements. Based on other studies, these chicks were considered to have a suboptimal "carryover" of unidentified chick growth factors. In

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TABLE I. Composition of Basal Rations* Used.

Ingredients	%
Corn-soybean meal type ration (A)	
Ground yellow corn	60.85
Soybean oil meal, solvent, 44% protein	34.
Limestone	1.
Bone meal	3.25
Sodium chloride, iodized	.3
Cod liver oil (2250 IU vit. A, 300 ICU vit. D/g)	.25
Manganese sulfate	.025
DL-methionine	.05
	mg/lb
Riboflavin	1.6
Niacin	8.
Calcium pantothenate	2.
Choline chloride	200.
2-methyl-1, 4-naphthoquinone	.2
Vit. B_{12}	.01
Oat groat-fish meal type ration (B)	
	%
Oat groats	83.
Fish meal	15.
Calcium carbonate	1.25
Sodium chloride, iodized	.3
Dry vit. A and D supplement (4000 IU vit. A, 750 ICU vit. D/g)	.2
Manganese sulfate	.02
Copper sulfate	.001
Cobalt sulfate	.0001
DL-methionine	.08
Glycine	.3
	mg/lb
Riboflavin	1.6
Niacin	16.
Calcium pantothenate	5.
Choline chloride	200.
2-methyl-1, 4-naphthoquinone	.2
Alphatocopherol acetate	3.
Pyridoxine HCl	1.6
Biotin	.025
Folacin	.2
p-aminobenzoic acid	10.
Vit. B_{12}	.002

* These rations are considered adequate in all known nutrients required by the chick. However, ration A is considered to supply suboptimal amounts of 2 different unidentified growth factors, one of which is supplied by fish meal(7). Ration B was designed to supply ample quantities of the unidentified growth factor present in fish meal but contained no soybean oil meal, which also has been reported to supply unidentified growth factor activity(8,9).

all other experiments, the chicks were progeny of dams maintained on litter and fed a complete breeder ration. Feed and water were allowed to be consumed *ad libitum*. In no instance was excessive mortality encountered. The day-old chicks were individually wing-banded, placed in electrically heated battery brooders with raised wire floors, then weighed at weekly intervals during a 4-week experimental period. All experiments were conducted in rooms previously used for rearing chicks. Bacteriological methods employed have been described previously(2). The composition of the two basal rations used is presented in Table I. Ration A was fed in Exp. 1 through 6 and ration B was used in Exp. 7 and 8. Various bacterial preparations, made as described below, were fed as supplements. In Exp. 1 the mash used was prepared by fermenting a chick growing mash with *A. aerogenes* isolated from the ceca of a four-week-old chick which had been fed procaine penicillin G. The fermentation was effected by incubating the inoculated substrate at 35°C for 3 days under anaerobic conditions. The solid and liquid portions of the substrate were then dried separately and recombined. The organism supplement used in this experiment was prepared by incubating a skimmilk culture of *A. aerogenes* for 24 hours at 35°C. Three hundred ml of the resulting ferment were then added to 200 g of sterile ground corn and air dried. In the remaining experiments, *A. aerogenes* was grown on the surface of Eugon agar (Baltimore Biological Laboratory) in Kolle flasks. After 24 hours at 37°C, the surface growth was removed from each flask with 12 ml of a 10% solution of sterile Difco skimmilk. The cell suspension was dried by lyophilization. Thus higher concentrations of viable cells were obtained. In Exp. 3, *E. coli* was grown in the same manner. In addition, *A. aerogenes* was also cultured in skimmilk for 24 hours at 37°C under aerobic conditions, after which the complete culture was lyophilized. In all experiments involving the mixtures of dried organisms and skimmilk solids, a level of 0.022% was added to the diet. This level supplied approximately 0.003% dried bacterial cells. The cultures were added to the feed at the beginning of each experiment and stored at 10°C in containers with tight fitting lids during the 4-week experimental period.

Results. The results obtained in Exp. 1 are presented in Fig. 1. The average weight of the basal group was 219 g. Growth responses were obtained when the fermented

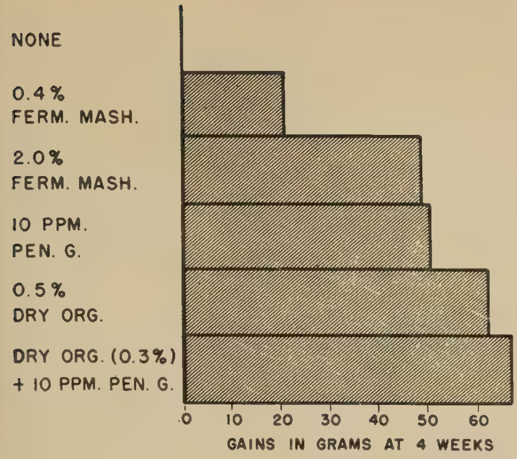

FIG. 1. Effect of *Aerobacter aerogenes* fermentation product and air dried bacterial cells on chick growth to 4 wk.

mash containing viable *A. aerogenes* was added to the diet. When the 2.0% level was used, the growth response was comparable to the addition of 10 ppm procaine penicillin G. When the preparation of dried organisms was added to the diet, alone or in combination with penicillin, slightly greater gains were observed. The relatively large gains noted here, as compared with those in the subsequent experiments, is believed to be due to the fact that the chicks used in this experiment had a lower "carry-over" of unidentified growth factors from their dams.

Exp. 2 was designed to determine the effectiveness of oral administration of lyophilized cultures of *A. aerogenes* and *E. coli,* alone and in combination with lactose and penicillin. The results of this experiment and number of viable organisms per gram of feed after 4 weeks storage are presented in Fig. 2. When lyophilized cultures of *A. aerogenes* or *A. aerogenes* plus *E. coli* were added to the basal diet without penicillin and lactose, the growth was only slightly improved. This improvement was equal to that obtained with procaine penicillin G when the culture of *E. coli* was fed. The chick growth response obtained when 3% lactose and 10 ppm procaine penicillin G were added to the basal diet is shown by the line bisecting the chart. When the cultures were fed in combination with penicillin and lactose chick growth was further stimulated, and in all cases exceeded that obtained with penicillin and lactose supplements alone. The results obtained by feeding lyophilized *A. aerogenes* and *E. coli* indicate that these viable cultures exerted a growth promoting effect particularly in the presence of the antibiotic.

In addition to the experiments described above, ration A was used in three other trials. Two experiments also have been performed in which ration B and *E. coli* cultures were employed. The results of all experiments are

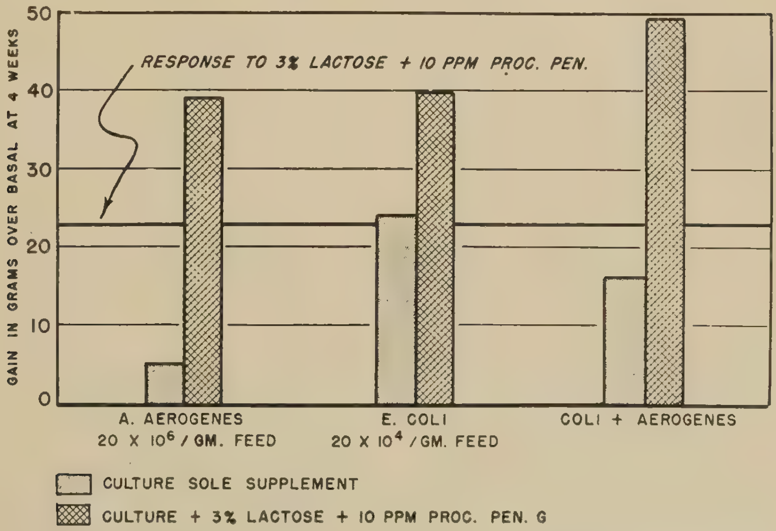

FIG. 2. Effect of lyophilized *Aerobacter aerogenes, Escherichia coli,* and their combination, with and without lactose and penicillin on chick growth to 4 wk.

summarized in Table II. Although the supplements containing viable organisms produced little growth response and in several instances seemed to cause a slight depression in growth when no antibiotic was fed, these cultures did improve the growth rate in every case when 10 ppm of procaine penicillin G were also fed. The average growth increase obtained with the addition of 10 ppm of procaine penicillin alone to rations A and B was 30 and 42 g, respectively. Further addition of viable organisms in the presence of the antibiotic resulted in average growth increases of 54 and 69 g over that obtained with basal rations A and B. Consequently, the growth promoting effect of the antibiotic was increased an average of 80 and 64% when viable cultures of *A. aerogenes* and/or *E. coli* were added to rations A and B. The average increase in chick weight in all 6 experiments, which resulted from the addition of cultures of viable organisms to ration A with added penicillin is statistically significant to the 1% level as measured by analysis of variance. These results reveal, therefore, that continuous feeding of certain viable penicillin-resistant bacterial cells was effective in promoting chick growth when procaine penicillin G was present in the ration.

TABLE II. Summary of Experiments Showing Effects of Viable Penicillin Resistant Microorganisms on Chick Growth to 4 Weeks with and without Antibiotic, 8 Exp.

			10 ppm procaine penicillin G	
Exp. No.	Without antibiotic; no added organisms	Organisms added	No added organisms	Organisms added
		Ration A		
1	219 (15)	282 (14)	270 (17)	285 (13)
2	312 (20)	328 (16)*	336 (19)	361 (16)
3	309 (27)	320 (14)† 321 (14)‡ 351 (16)§	349 (15)	384 (13) 384 (14) 353 (15)
4	304 (18)	284 (18)	318 (20)	355 (17)
5	310 (18)	319 (20)	353 (20)	358 (20)
6	345 (18)	329 (18)	350 (18)	361 (14)
Avg	299	318	329	353
		Ration B		
7	261 (17)	267 (17)‖	307 (15)	333 (17)
8	268 (18)	258 (19)‖	307 (19)	335 (18)
Avg	265	263	307	334

* 0.022% each of lyophilized *E. coli* and *A. aerogenes* obtained from Kolle flask surfaces was added to diet.

† A lyophilized *A. aerogenes* culture obtained from Kolle flask surfaces was added to diet.

‡ A lyophilized skimmilk culture of *A. aerogenes* which contained 100 u of procaine penicillin G prior to fermentation added to diet.

§ A lyophilized skimmilk culture of *A. aerogenes* which contained no antibiotic prior to fermentation added to diet.

‖ Diet supplemented with a lyophilized culture of *E. coli* obtained from Kolle flask surfaces.

Figures in parentheses refer to No. of surviving chicks.

Discussion. Anderson, Slinger, and Pepper (6) have also obtained preliminary results similar to some of the observations reported previously(5) and in this paper. These investigators fed nutrient broth cultures of a mutant strain of *E. coli* to chicks and poults, adding them daily to the feed. The *E. coli* cultures promoted significant growth increases when fed in the presence of penicillin.

Continuous feeding of certain viable bacterial cultures to chicks accentuates the growth-stimulatory action of orally administered procaine penicillin G. This strongly indicates that the bacterial population in the intestinal tract exerts a marked effect on antibiotic function. The finding that antibiotics have no growth promoting properties when fed to chicks reared in germ-free environment(10) or in environments not used previously for rearing chicks(11,12) also support this view.

Although both *A. aerogenes* and *E. coli* are known to synthesize certain nutrients, one cannot conclude from these results that intestinal synthesis of unidentified nutrients is necessarily involved. Since significant stimulation in growth was obtained with cultures supplying such small amounts of dry matter, it is felt that the results obtained were due to effects of the viable organisms in the intestine rather than to the presence of unidentified nutrients in the cultures fed. Jones and Combs(13) reported that either penicillin or aureomycin were effective in sparing the dietary requirement of chicks for unidentified growth factors. Scott and Jensen(14) have reported recently that aureomycin appeared to spare the requirement of poults for an unidentified growth factor not present in fish meal to a greater extent than for the unidentified factor supplied by fish meal. It is also possible that the continuous feeding of *A. aerogenes* and *E. coli* materially aided the antibiotic in reducing the numbers of less desirable organisms in the intestinal tract, thereby permitting more rapid chick growth.

Summary. Pure cultures of *E. coli* and *A. aerogenes* were grown, lyophilized, and fed to chicks as dietary supplements both in the presence and in the absence of procaine penicillin G. Little or no chick growth response was obtained when either of these organisms were added to the ration in the absence of the antibiotic. Greater gains were obtained when 10 ppm procaine penicillin G were fed. When viable cultures of *A. aerogenes* and *E. coli* were fed in combination with penicillin, growth was further increased significantly. The effectiveness of the antibiotic in promoting chick growth was increased 64 and 80% when these organisms were added to the feed. The results obtained illustrate the influence of bacterial environment on the antibiotic growth effect and in nutritional studies.

The authors are indebted to Lederle Laboratories, Pearl River, N. Y., for providing folacin; Merck and Co., Rahway, N. J., for other crystalline vitamins; and U. S. Industrial Chemicals, for DL-methionine and for preparing the fermented mash used in Exp. 1.

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Skim Milk Powders and Experimental Rat Caries. (20252)

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In a previous publication(1) it was reported that a diet containing 4 heat-processed cereal foods and 18% cerelose produced dental caries in white rats. The results were important because of the type of caries which developed on the smooth surfaces of the teeth, and because neither an excessive quantity of sugar nor coarse food particles were implicated as cariogenic factors. Moreover, the results suggest that serious attention should be given to the possibility of an effect of nutritive deterioration which may accompany the heat-processing of foods, on the etiology of experimental dental caries in white rats.

Perhaps no constituent of the diet has been more widely studied for the effects of heat-processing, than have the skim milk powders. Such studies are of obvious importance when it is realzed that in 1951 approximately 700,000,000 lb of skim milk powder, and 130,000,000 lb of whole milk powder were produced in the United States for human consumption, particularly in bakery products, confectioneries and ice creams(2). In general, there is adequate evidence in numerous previous studies that skim milk powders during preparation and storage undergo nutritive losses in the protein fraction, as shown by effects on growth, digestibility and biological value(3-7). However, none of the published results of studies on nutritive properties of skim milk powders *per se* have reported observations on dental caries. In one prior abstract report on dental caries in white rats, placed on "low fat, high sugar diets consisting chiefly of skimmed milk (powder) and cane sugar, supplemented with cod liver oil," surface caries were observed "on the buccal and lingual surfaces of both the upper and lower molar teeth of albino rats in addition to proximal surface and occlusal fissure caries"(8). The Syrian hamster also may develop a cervical type of dental lesion on buccal and lingual surfaces when fed a diet composed essentially of cornstarch and whole milk powder(9). In these and other prior caries studies the cariogenic role of milk powders in the diets may have an added significance in the light of the results of this experiment. The incrimination of heat-processing or storage deterioration of foods such as cereals and milk powders, in the production of dental caries is a relatively new approach in experimental caries research.

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Materials and plan. The primary object of these experiments was to study the cariogenic properties of several dry skim milk powders as obtained from commercial sources, and to determine the effect of an additional heat-treatment on their cariogenic action. One spray-process and 2 roller-process powders were studied before and after being given an additional heat-treatment. The milk powders were present at a 35% level in all the diets. The other components of the diets were: 18% glucose (commercial cerelose), 45.0% cornstarch and 2.0% dehydrated liver (excepting Diet 598 which contained 63.0% cerelose and no cornstarch). A concentrated vit. A, D and E supplement was given by mouth weekly. The diets were finely powdered before they were fed.

The analysis of the diets was as follows:

Diet No.		598	614	615	616	633	634	635	636
Protein (Nx 6.25),	%	13.56	14.31	13.68	13.56	13.25	13.50	13.56	13.75
Ash	%	3.04	3.04	3.02	3.04	2.83	2.92	3.01	2.92
Calcium	%	.48	.46	.44	.44	.44	.43	.47	.46
Phosphorus	%	.38	.39	.38	.39	.36	.36	.35	.36
Fluorine (ppm)		.4	.5	.5	.5	.9	.9	1.0	1.0

Considering the lactose present in skim milk powder, the total lactose plus cerelose content of Diet 598 was 81.2%; the other diets 36.2%. The milk powders present in the diets and the heat treatments were as follows: *Powder A* was an improved low-heat spray-process, non-fat dry milk powder. *Powder B* was prepared by placing powder A about one-half inch deep in a pan sealed with adhesive tape(5) and heating in an autoclave for 15 minutes at approximately 121°C. *Powder C* was a roller process non-fat dry milk powder from the same commercial source as powder A. *Powder D* was prepared from powder C by the same heat treatment described for powder B. *Powder E* was a roller process powder obtained from a commercial source different from powder C. *Powder F* was prepared by placing powder E one to 1½ inches deep in a sealed pan and heating in an autoclave 15 minutes at approximately 121°C. *Powder G* was prepared by placing powder E one to 1½ inches deep in an open pan and heating in an autoclave 15 minutes at 15 lb. Milk powders B & D, heated in closed pans, were subjected to essentially a dry heating and were a pale cream color only slightly darker than the unheated powders from which they were prepared. Milk powder G, which was heated in an open pan received the full effect of the moist heat of autoclaving and was a dark brown color. This additional heat-treatment may be compared in some respects to heating processes required for the preparation of certain commercial foods which contain skim milk powders. The *moisture and nitrogen* contents of the raw milk powders were respectively 3.83% and 5.61%, powder

TABLE I. Results of Feeding Diets Containing Heated and Unheated Skim Milk Powders on the Caries Experience of Young White Rats.

Exp. No.	1	2	3	4	5	6	7	8	9
Diet No.	633	634	635	636	598	614	615	616	616
Skim milk powder	A	B	C	D	E	E	F	G	G
" " " heated	—	+	—	+	—	—	+	+	+
No. of rats	40	40	39	38	35	40	39	39	30
" " litters	34	33	30	29	7	17	17	8	22
Days on exp.	90	90	90	90	91	91	91	35	42
Initial wt	26.7	26.9	26.3	26.2	31.6	28.2	28.2	27.5	27.8
Final "	216.7	83.6	199.1	46.7	163.2	193.2	178.8	23.5	23.6
Avg daily gain	2.11	.63	1.92	.23	1.45	1.81	1.65	–.10	–.10
					Caries incidence—%				
Carious rats	12.5	40.0	51.3	84.2	48.6	57.5	74.4	56.4	23.3
Occlusal carious rats	.0	.0	7.7	5.3	8.5	.0	5.1	.0	.0
Surface " "	12.5	40.0	43.6	84.2	42.9	57.5	71.8	56.4	23.3
					Caries distribution and severity—No./rat				
Upper carious teeth	.00	.08	.03	.13	.09	.00	.10	.00	.00
Lower " "	.20	1.03	1.00	2.82	1.40	1.35	2.05	1.69	.57
Total " "	.20	1.10	1.03	2.95	1.49	1.35	2.15	1.69	.57
Occlusal carious teeth	.00	.00	.15	.11	.29	.00	.15	.00	.00
Surface " "	.20	.10	.87	2.89	1.23	1.35	1.97	1.69	.57
Occlusal carious areas	.00	.00	.21	.13	.31	.00	.15	.00	.00
Lingual " "	.00	.00	.00	.05	.09	.00	.00	.00	.00
Buccal " "	.28	1.78	1.15	4.08	1.89	1.93	3.03	2.64	.60
Score/rat	.30	2.63	2.72	7.11	2.57	2.15	3.95	2.85	.67
Score/carious rat	2.40	6.56	5.30	8.44	5.29	3.74	5.31	5.05	2.86

A; 3.31% and 5.63%, powder C; and 4.13% and 5.24%, powder E. Powders A, B, C, and D were kept at approximately 5°C as were all the diets; powders E, F, and G, were stored in a constant temperature room at 20°C. *Two strains of rats* were used in these studies, Sprague-Dawley in Exp. 5, 6, 7, and 8, Holtzman in Exp. 9, and both strains in Exp. 1, 2, 3, and 4. In the latter 4 experiments the results on both strains were essentially the same and the data were pooled to obtain the final results. Litter mates were used to compare heated *vs* unheated milk powders and for the comparison of powder A *vs* powder C. The rats were started at weaning age, were fed *ad libitum* in screen-bottom cages (2 rats per cage) and were given distilled water. At the end of 90 or 91 days, or when the rats died, as in Exp. 8 and 9, the heads were autoclaved, cleaned of adhering soft tissues, and the teeth, *in situ,* kept under water in the cold. The dental examinations were made with a low-power microscope, starting with the teeth in the moist state and continuing while the tooth surfaces dried in the heat of the microscope lamp. The dental lesions were recorded and scored as follows: occlusal, lingual and buccal surfaces were divided into two or three areas (interproximal surfaces were divided between lingual and buccal surfaces) and each carious defect was scored one, 2, or 3, according to the extent of the carious destruction. As has been mentioned previously(1) the initial surface-caries process was observed as a white, opaque, usually elongated area, following most generally the line of the gingival attachment. Frequently these opaque areas were small and did not seem to penetrate the dentin; in other instances a very considerable destruction of both enamel and dentin occurred. The final diagnosis of the dental defects included 3 essential items as shown in Table I, *i.e.,* a) the per cent of affected rats in the group, b) the number and location of the affected teeth, and c) the severity of the caries as indicated by the number of surface areas involved and the caries score. It should be emphasized that our evaluation of the data on the experimental caries diagnosis includes all 3 of the above essential items.

Results. The combined data on the caries

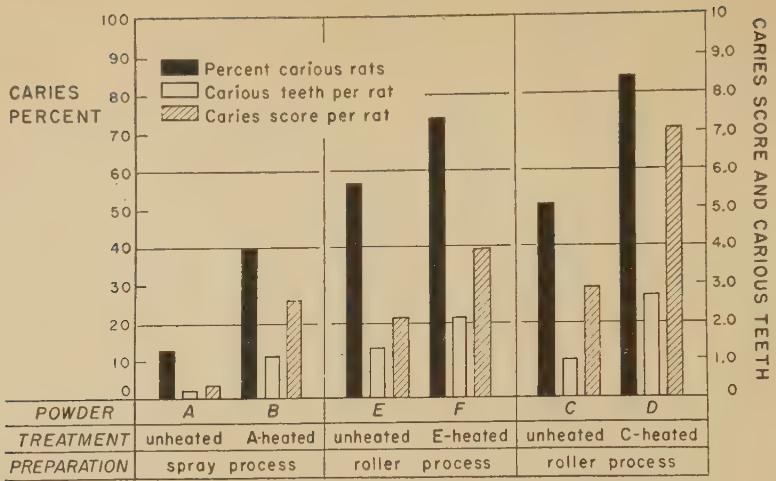

FIG. 1. Incidence and severity of dental caries in white rats fed diets containing heated and unheated dry skim milk powders.

diagnosis and growth effects of the various diets are shown in Table I. The most serious growth failures occurred in Exp. 8 and 9, in which the milk powders were most drastically heat-treated. All the rats in these 2 experiments died by the end of 45 days. Diets 634 and 636 were both inadequate for growth although Diet 634 promoted slightly better growth than Diet 636. Of the unheated milk powders, powder A proved most satisfactory for growth, powder E unheated in Diet 614 and heated in Diet 615, promoted better growth than in Exp. 5, Diet 598. The explanation of differences in growth resulting from heat-treated milk powders seems to relate to the severity of the heat-treatment. The depth of the milk powder in the pan or other unrecognized variations during heating, may have influenced the heat-effects and explain the much better growth results obtained with powder F (Diet 615) *vs* powder D (Diet 636).

The cariogenic effects of the 3 unheated milk powders were most pronounced for powder C (Diet 635) and powder E (Diet 614 and Diet 598), all of which were prepared commercially by the roller-process, whereas powder A, the low-heat, spray-process powder used in Diet 633, was only slightly cariogenic (Table I). There is considerable uniformity in the results obtained with the two roller-process powders C and E, as fed in Diets 598, 614 and 635. It is of interest that these similar effects occurred when as much as 67% cerelose was present in Diet 598 as compared with only 18% cerelose in Diets 614 and 635. A direct comparative study of the effects of heat-treatment on the milk powders was made in Exp. 6 (powder E) *vs* Exp. 7 (powder F); Exp. 1 (powder A) *vs* Exp. 2 (powder B); and Exp. 3 (powder C) *vs* Exp. 4 (powder D). In all these three trials of milk powders, A, C, and E, heated and unheated, a greater incidence of caries and more severe caries resulted from the heated powders (Table I and Fig. 1). This enhanced cariogenic effect is particularly apparent in the comparison of unheated powders A and C *vs* heated powders B and D.

The results with Diet 616 (heated powder G) are somewhat contrary to the results with other heated powders although it is possible that the early death of these rats which permitted only a 35-42-day feeding period caused this limited development of caries.

A survey of the caries data shown in Table I brings out the pertinent fact that the majority of the carious areas occurred in the lower teeth, and on buccal surfaces and that there were practically no carious areas on the occlusal or lingual surfaces. This result is a notable contrast to our previous observations that coarse particle corn meal diets(10) and purified high sugar diets(11) produce only a fissure type caries on occlusal surfaces. In some respects it would appear that the surface type caries rather than the occlusal fissure caries in experimental animals bears the closest resemblance to human dental caries (1,9).

Discussion. The results of these experiments agree with our previous evidence(1) that a nutritive deterioration of certain processed cereal foods, as brought about in their commercial preparation or by heat-treatment may be associated with the production of carious defects in white rats' molar teeth. In these continuing studies with skim milk powders, the same specific type of surface carious defects have been produced, and there appears to be every reason to incriminate an effect of heating skim milk powders, as one causative factor. The significant difference in the dental results obtained with milk powder A (Diet 633), a low heat spray-process powder *vs* the 2 roller-process powders C (Diet 635) and E (Diets 614 and 598) seem to relate a causative factor with the method of preparation of the 2 types of powders.‡ The spray-process powder was definitely less cariogenic, than the 2 roller-process powders. Substantial evidence that an additional heat-treatment of the 3 milk powders aggravated their cariogenic properties is present in the 3 comparable studies of these powders before and after heating (Table I and Fig. 1). In each of these 3 comparisons the diets containing the heat-treated powders were more cariogenic than the comparable unheated powders.

The nutritive losses which occur in skim milk powders are generally attributed to highly complex chemical changes in the milk proteins. There is conclusive evidence of a major loss of lysine and also evidence of some partial destruction or inactivation of several other essential amino acids(3,6,7,12). Our observed failures in growth are more than likely due to these well-established changes in milk protein values. However, there is no evidence which permits at this time any identification of such changes in protein values with the production of the carious lesions observed in these experimental rats. The role of cerelose as a causative factor is certainly not suggested and mineral and vitamin deficiencies seem unlikely causative agents at this time. Variations in dietary fluorine do not explain the caries differences. It should not be overlooked nevertheless, that any attempt to explain the relation of a dietary factor to dental caries etiology ordinarily cannot avoid considering saliva chemistry, the oral flora, the oral tooth surfaces and both the organic and inorganic chemistry of the teeth.

A comparison of the caries data with the growth data for powders A and C suggest that the cariogenic activity of these powders may not be related to their ability to promote normal growth. Thus the rats given powder C gained only slightly less than their litter mates on powder A, but the rats on powder C had more than 4 times as much caries, and according to average scores, their caries was almost 10 times as severe as that of rats given powder A. Similarly, the growth differences in rats given powders E and F are not accompanied by comparable differences between the two groups in caries incidence or severity.

These results on growth as compared with cariogenicity seem to suggest therefore that the cariogenic factor is possibly not the same as the growth inhibiting factors. Perhaps some additional support of this idea is contained in the observation that heat-processing of powder A (*i.e.* powder B) caused a drastic failure in growth but not a correspondingly high incidence of caries, nor was caries as severe as occurred from powder C. A question regarding the nature of the changes which occurred in preparing powders A and C and the subsequent heat-treatments is raised by the fact that powder B was not as cariogenic as powder D although both were heat-treated powders. Likewise powder B although heat-treated was no more cariogenic than the unheated roller-process powder C.

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‡ According to the Bureau of Dairy Industry, U.S.D.A. (BDIM-Inf. 25, 1949) dried milks made by the spray-process are finely divided, very dispersible in water, and hydroscopic, while in contrast, because of the high temperatures necessary for drying in the roller-process, there may be a slight discoloration of the product and a partial coagulation of the proteins which makes them "insoluble." The roller powder is practically nonhydroscopic. The roller process is used mainly in the drying of skim milk and buttermilk, while the majority of whole milk powder is prepared by the spray-process.

Finally it may be pointed out that additional studies are under way not only to further evaluate the relation of varying heat-treatments to cariogenicity, but to study also the effects of storage of milk powders under controlled conditions of temperature and humidity. As in previous observations on experimental rat caries, caution is indicated in drawing conclusions in the light of the unresolved variations in dental caries susceptibilities which occur among different strains of rats and in different research laboratories. Litter susceptibilities to caries varied in these studies as in previous studies(1,10,11). These results, it should be emphasized, were obtained with two strains of rats, the Holtzman and Sprague-Dawley, as produced in the stock colony maintained at the National Institutes of Health.

The effects of heat-treatment on the development of caries gains added emphasis however when it is realized that in all these 3 experiments the rats compared with litter mates, and the diets were identical in all respects except the source and method of preparation of the milk powders and their subsequent heat-treatment.

Summary. Previous studies on experimental rat caries suggested the possibility of a relation between heat-processed cereal foods and dental caries etiology. Continuing this type of study, the cariogenic role of heat-processed skim milk powders, has been investigated. Diets containing 35% of commercial skim milk powders prepared by the usual spray and roller processing and also given a subsequent heat-treatment prior to feeding, have been identified with the production of a specific type of surface caries in white rats. The extent and severity of the caries was significantly and consistently increased by heat-treatment of these commercial skim milk powders. The results suggest that a cariogenic effect may be produced by heat-treatment of dry skim milk powders. According to present evidence this cariogenic effect has not been identified with the nutritive growth failures usually associated with heat or storage deterioration of milk proteins.

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Steroid Compounds Resulting from Incubation of Cortisone with Surviving Liver Slices.*† (20253)

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The major site of inactivation of steroid hormones is the liver. Catabolism of androgenic and estrogenic steroids by hepatic tissue has been demonstrated both *in vivo* and *in vitro*(1-3). The hepatic metabolism of several adrenocortical steroids has been investigated by incubating these compounds with liver slices(4). The results show that the steroids studied, including cortisone (Compound E), are catabolized by reduction of the conjugated, unsaturated bonds in ring A of the molecule and by degradation of the α-ketol side chain. These alterations in the molecular structure of cortisone result in loss of biologic activity of this hormone. Hechter (5) perfused the isolated rat liver with blood containing cortisone and observed rapid disappearance of this steroid. This observation, coupled with the clinical finding that Compound E administered orally is metabolically active, led this investigator to suggest that certain metabolites of cortisone, formed by the liver, must possess physiologic activity.

The purposes of the present investigation were to study the metabolism of cortisone by rat liver slices and to attempt identification of the compounds derived under the conditions of these experiments. The procedure followed included incubating rat liver slices in a physiologic medium containing cortisone and analyzing the resulting steroid mixture by paper chromatography. The results show that under the conditions of this experiment, two derivatives of cortisone are formed. One has been demonstrated to be Compound F and the other, which has been only partially identified, is thought to be Reichstein's Substance E.

Procedure. Rat liver slices weighing approximately 300 mg were placed in a flask containing 4.6 ml of Kreb's solution, buffered with phosphate to pH 7.4. Prior to incubation, 1.0 mg of cortisone (free alcohol) in 0.2 ml of propylene glycol was added. The flask was flooded with oxygen, stoppered, and incubated at 37° for 3 hours with occasional shaking. Control studies were done in which liver slices were incubated in the same medium but without the addition of cortisone. In addition, liver slices, which had previously been boiled in isotonic saline, were incubated in a medium containing cortisone. On completion of incubation, the steroid fraction was recovered by the following procedure. Absolute ethyl alcohol was added to the incubation mixture in amount sufficient to bring the final alcohol concentration to 70%. The mixture was allowed to stand overnight at room temperature and was filtered. The alcoholic solution was extracted 3 times with equal volumes of petroleum ether and the petroleum ether fraction discarded. The alcohol was removed by distillation *in vacuo* at a temperature of 45°C. The aqueous solution was extracted 3 times with equal volumes of ethylene dichloride, the latter fraction dried over sodium sulfate, and the ethylene dichloride removed under vacuum. The residue containing the steroids was dissolved in 1.0 ml of an equal mixture of chloroform and methyl alcohol and applied to the paper strip. Chromatography was done by the method of Zaffaroni(6) using propylene glycol and toluene as solvents. The steroids present on the chromatogram were detected by use of the alkaline silver nitrate reagent and by exposing the paper to ultraviolet light (2537A). Steroid compounds which reduce alkaline silver nitrate have an alpha-ketol side chain(6). Those steroids with conjugated, unsaturated bonds in ring A absorb ultraviolet light(7).

Identification of steroid compounds isolated. A variety of physical and chemical procedures

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were used in an attempt to determine the molecular configuration of the steroids isolated in this experiment. The following procedures were adapted for use in efforts to identify the 3 substances obtained. 1) *Ultraviolet absorption spectra.* A sample of each compound was dissolved in methyl alcohol and the absorption spectrum was determined from 226 to 250 mμ. Peak absorption at 240 mμ was considered evidence of an alpha-beta unsaturated ketone group(8). 2) *Absorption spectra of the sulfuric acid derivatives.* The steroids isolated in this experiment were treated with concentrated sulfuric acid and the spectra were compared with those obtained from the addition of sulfuric acid to crystalline Compound E and Compound F. Comparison was also made with spectral analyses of related steroids recorded in the literature(9). 3) *Comparison of chromatographic mobility of unknown steroids with that of crystalline compound E and compound F.* Aliquots of the unknown steroids were chromatographed with crystalline samples of cortisone or Compound F. 4) *Color reactions.* The unknown substances present on the chromatograms were tested with certain color reagents as follows: a) Alkaline silver nitrate (6), b) Iodine reagent(8), c) Concentrated sulfuric acid(8).

Results. Control observations demonstrate that no steroid compound was present on the chromatogram when the incubation medium did not contain cortisone (Fig. 1, A). In addition, a single band identified as cortisone was obtained on chromatograms when liver slices had been previously inactivated by boiling and were incubated in a medium containing cortisone (Fig. I, B).

In all other experiments, when liver slices were incubated in a medium containing cortisone, 3 distinct fractions were detected (Fig. 1, C). For purposes of discussion, these are designated as incubation products I, II, and III in descending order. Areas of the paper chromatogram containing these products were separately eluted and efforts to identify each substance were made by the procedures described above.

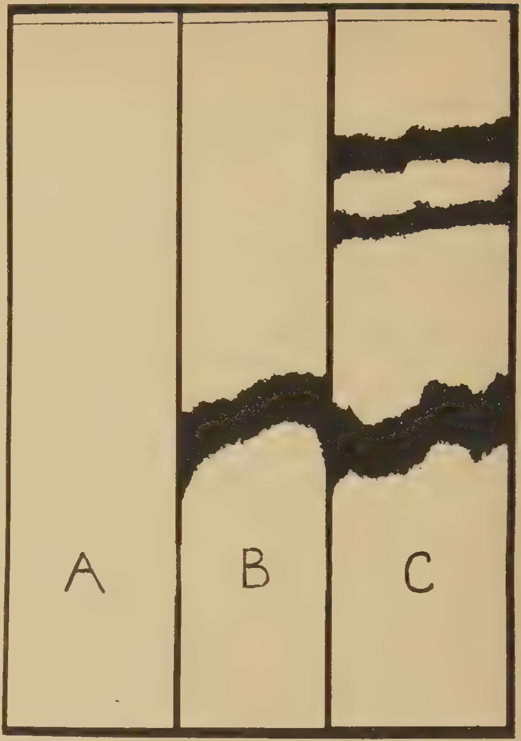

FIG. 1. Steroid compounds obtained on paper chromatograms. Dark bands represent steroids which absorb ultraviolet light. A. Chromatogram of surviving liver slices incubated in a medium without cortisone. B. Chromatogram of inactivated liver slices incubated in a medium containing cortisone. C. Chromatogram of surviving liver slices incubated in a medium containing cortisone.

In all experiments, incubation product III was obtained in greatest amount as judged by size and intensity of the band when the paper chromatogram was visualized under ultraviolet light. The following results establish the identity of this compound as cortisone: 1) a positive reaction for an alpha-ketol group when tested with alkaline silver nitrate, 2) an intense blue color with the iodine reagent, 3) maximum ultraviolet absorption at 240 mμ, 4) chromatographic mobility identical with that of crystalline cortisone, and 5) a sulfuric acid derivative with absorption maxima at 280, 345, and 415 mμ. These absorption peaks coincide with those of the sulfuric acid derivative of crystalline cortisone.

Incubation product II was proven to be Compound F. Results of procedures designating the molecular structure of this steroid included: 1) a positive reaction for an alpha-ketol side chain with alkaline silver nitrate, 2) production of a compound with yellow-green fluorescence when treated with concentrated sulfuric acid, 3) maximum absorption at 240 mμ in the ultraviolet, 4) chromatographic mobility identical with that of compound F in mixed chromatograms, and 5) addition of sulfuric acid produced a colored product with absorption maxima at 280, 395, and 475 mμ. This is identical with the spectrum derived from crystalline Compound F.

Total identification of the molecular configuration of incubation product I was not achieved largely because the quantity of this material obtained was limited. The data available showed that 1) this compound did not reduce alkaline silver nitrate and, thus, does not possess an alpha-ketol side chain; 2) a maximum absorption at 240 mμ indicated that the alpha-beta unsaturated ketone was present; 3) the chromatographic mobility was less than that of cortisone or Compound F. The decreased mobility of this product as compared to Compound F could be accounted for either by reduction of the ketone group on the carbon atom at position 20 to an alcohol or by the addition of another atom of oxygen to the molecule. It is suggested but not established that the substance is Reichstein's Substance E ($\triangle$4-pregnene-11 β, 17 α, 20, 21 tetrol-3-one) and is formed from cortisone by reduction of the ketone groups at C-11 and C-20.

Discussion. The data presented by Schneider and Horstman(4) show that liver slices alter the molecular structure of cortisone by reduction of the conjugated, unsaturated bonds in ring A and by degradation of the side chain at C-17. The studies reported here did not concern these changes but demonstrate that rat liver slices may also metabolize cortisone by reducing the ketone groups at C-11 and probably at C-20. Conversion of the ketone at position 11 to an hydroxyl group results in the formation of Compound F. If both the carbonyl groups at C-11 and C-20 are reduced the steroid formed is Reichstein's Substance E. This compound has been isolated from the adrenal cortex(10).

It has been demonstrated that conversion of Compound E to Compound F occurs *in vivo* and it has been suggested that this may account for some of the metabolic activity previously attributed to cortisone. Burton, Keutmann, and Waterhouse(11) administered cortisone acetate to normal humans and patients with Addison's disease and recovered increased amounts of cortisone, Compound F, tetrahydrocortisone, and a fourth steroid from the urine. Hechter(5) has pointed out that although cortisone is rapidly destroyed by the liver, the metabolic activity following oral administration of this steroid persists for some time. This investigator has suggested that certain metabolites of cortisone formed in the liver are physiologically active. Ingle(12) has observed that cortisone does not exert its usual effect on carbohydrate tolerance in the hepatectomized rat, whereas the administration of aqueous adrenal cortex extract results in a decrease in glucose tolerance. Since adrenal cortex extract contains a variety of steroid hormones, including Compound F, this observation seems to indicate that cortisone must be altered by passage through the liver before it can affect carbohydrate metabolism.

These observations taken together with the experimental data reported here which demonstrate that cortisone is partially converted to Compound F by liver slices suggest that some of the metabolic activity which in the past has been attributed to Compound E may actually be dependent on its conversion to Compound F.

Summary. 1. Liver slices were incubated in a physiologic, buffered medium containing cortisone. Following incubation, the steroid fraction was recovered and the individual steroids isolated by paper chromatography. 2. Under the conditions of this experiment, 3 steroids were isolated on the paper chromatogram. The compound of greatest mobility and obtained in largest amount was cortisone, unaltered by incubation. The product of intermediate mobility was identified as Compound F. The least mobile compound was not identified. 3. On the basis of observations in the literature and the experimental data reported here, it is suggested that the metabolic activity of cortisone may actually be dependent on its conversion to Compound F.

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Some Effects of Desoxycorticosterone Glycoside on Chick Fibroblasts *in vitro*.* (20254)

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Although desoxycorticosterone is known to elicit a definite response from connective tissues when injected into the intact body, the effect of this substance upon connective tissues *in vitro* is comparatively little known. Adrenal cortical extracts have been reported by von Haam and Cappel(1), to prolong the survival of fibroblasts grown in Carrel flasks. Using desoxycorticosterone at concentrations of 0.03 mg/ml, Cornman(2) showed that there was a reversible inhibition of the beat of heart fragments taken from chick embryos; he also showed that fibroblasts are selectively damaged in mixed cultures of fibroblasts and endothelium grown in roller tubes. The investigation reported here was undertaken to study the effects of low concentrations of desoxycorticosterone on the rate of growth and the morphology of cells in tissue culture *in vitro*.

Materials and procedures. Stock cultures of connective tissue cells in a medium of chicken plasma and embryo extract were derived from explanted fragments of 7-day-old chick femurs. The cultures were cut into fragments as nearly equal in size as possible. One fragment was explanted into a medium consisting of equal parts of heparinized chicken plasma and chick embryo extract, the latter containing varying amounts of desoxycorticosterone glycoside.† The control fragment was explanted into the same medium without the desoxycorticosterone. The final concentrations of the desoxycorticosterone glycoside in the medium were: 30, 15, 7.5, 3.5, and 2 μg/ml. For each concentration about 20 pairs of cultures were made. Some other cultures were studied with this steroid at concentrations up to 240 μg/ml. The areas of outgrowth of the cultures were measured after 48 hours and the relative increases treated statistically(4). At the same time, photomicrographs were taken of cells in selected pairs of cultures to record morphological changes.

Results. Concentrations of desoxycorticosterone glycoside above 30 μg/ml were found to decrease the growth rate of tissue cultures of chick embryo fibroblasts. Below this concentration the steroid increases the rate of growth of fibroblasts. The stimulation of the growth rate of fibroblasts increases with decreasing concentration until a maximum stimulation is reached at 7.5 μg/ml. Below this concentration, the stimulation decreases until, at 2 μg/ml it is no longer measurable by these methods. The average differences in the relative growth of experimental and con-

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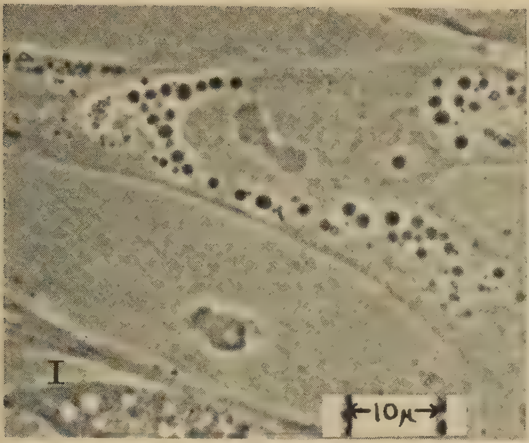
* This work was done under Contract with the Office of Naval Research.

† Obtained through the courtesy of Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Summit, N. J.



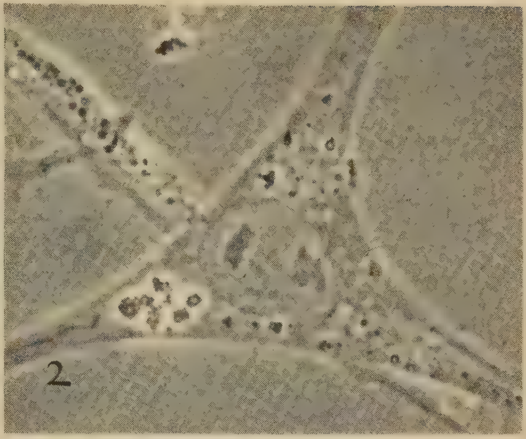


FIG. 1. Normal, untreated chick connective tissue cell in tissue culture *in vitro*. Living; phase microscopy. Distance between two dark bars in rectangular white area is 10 μ.

FIG. 2. Chick connective tissue cell in culture containing 7.5 mg/ml of desoxycorticosterone glycoside. Note reduction of cytoplasmic lipoid material. Living; phase microscopy. Same magnification as in Fig. 1.

trol cultures (based upon about 20 pairs for each concentration) were as follows: 30 μg/ml, –6.2; 15 μg/ml, 2.4; 7.5 μg/ml, 6.3; 3.5 μg/ml, 2.2; 2 μg/ml, 0.0.

Structurally, cells treated with concentrations of desoxycorticosterone glycoside above 30 μg/ml were characterized by enlarged nuclei and an increase in the rate of accumulation of fat droplets in the cytoplasm. At the highest concentrations used, (240 μg/ml) the nuclear membranes appeared to have been ruptured. Below 30 μg/ml photomicrographs show that the fibroblasts accumulated lipids in the cytoplasm at a much reduced rate (Fig. 1 and 2). At these concentrations there were few, if any, enlarged nuclei.

Summary. Concentrations of 15 μg/ml or lower of desoxycorticosterone glycoside stimulate growth of chick embryo fibroblasts and decrease the accumulation of cytoplasmic lipids. Concentrations of 30 μg/ml reduce growth and damage the nuclei of the cells.

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Action of Cortisone on Developing Chick Embryo.* (20255)

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This study was undertaken to determine whether the growth inhibition produced by cortisone is generalized or is limited to certain organs or regions. Previous studies on chick embryos(3,4) and on newly-hatched chicks (2) have emphasized growth-inhibition following injection of cortisone. In the present experiments, effects of cortisone are evidenced by a proportionate weight reduction of all parts tested except for the liver (smaller), eyes (larger), and gonads (larger), when com-

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* This investigation was supported by a research grant from The National Heart Institute, of the National Institutes of Health, Public Health Service.

TABLE I. Relative Body and Organ Weight in Control and Cortisone-Treated Embryos.

	Saline controls ± S.E.[*]	.5 mg cortisone ± S.E.[*]	1.0 mg cortisone ± S.E.[*]
Body wt in g	23.7 ± .9	22.1 ± 1.1	13.6 ± 1.6
Liver[†]	2322.1 ± 46.5	2057.5 ± 59.8	1598.2 ± 91.2
Heart	730.4 ± 30.5	745.5 ± 26.9	803.5 ± 82.6
Spleen	37.5 ± 2.8	38.4 ± 2.3	35.7 ± 4.9
Ovary	23.8 ± 2.0	27.0 ± 3.2	59.1 ± 9.8
Testes	23.4 ± 2.6	22.2 ± 2.3	35.8 ± 3.5
Tibia	754.6 ± 51.1	741.0 ± 27.5	654.2 ± 34.4
Eye	1879.0 ± 73.1	—	2666.0 ± 163.6

[*] Stand. error calculated from $\sqrt{\frac{Sd^2}{n(n-1)}}$.

[†] All organ wt figures expressed as mg/100 g body wt.

parisons are made on the basis of milligrams of organ weight per 100 g of body weight. There is a retention of water in the cortisone-treated chick embryo.

Materials and methods. Chick embryos of 8 days of incubation were treated with a single dose of cortisone acetate (Merck) injected onto the chorioallantoic membrane. The Merck preparation of cortisone was diluted with normal saline so that one-half or one milligram of cortisone acetate was suspended in 0.3 ml of fluid. Embryos similarly injected with 0.3 ml of normal saline were used for controls. Benzyl alcohol is used as a preservative in the cortisone suspension, but controls injected with this alcohol were not considered necessary, since Karnofsky *et al.* (3) found that the amount of benzyl alcohol contained in a milligram dose of cortisone is far below the threshold for producing detectable effects on the chick embryo. To compare the relative sizes of organs, experimental series which had received one-half or one milligram of cortisone and saline-injected controls were sacrificed at 18 days of incubation. The wet weights of the whole embryo, liver, spleen, heart, and tibia were determined. The gonads were fixed *in situ* in Bouin's fluid, washed in 70% alcohol, dissected and weighed as fixed tissues from 70% alcohol. For purposes of comparison, all weights are expressed as milligrams of tissue per 100 g of body weight. Other series were treated with one mg of cortisone and sacrificed at daily intervals from 10 through 18 days of incubation. The lengths of the embryo, neck, forelimb and hindlimb were measured. The embryo was extended by placing it against a straight edge and body length was taken as the distance from the crown to the tail tip. Neck measurements were taken from the base of the skull to the pectoral girdle. Limb measurements were made by straightening the limb and measuring from the body wall to the tip of the longest digit. Wet and dry weights were used in calculating the percent of water in the embryo. To determine dry weight, embryos were dried in a hot-air oven to constant weight.

Results. While cortisone inhibited the growth of all parts of the embryo, not all organs of the dwarfed embryos were affected proportionately. Of the organs weighed, the liver, gonads and eyes showed an effect. The weights of heart, spleen and tibia were not significantly different from the controls when compared on the basis of organ weight per 100 g of body weight. Because of their recessed position, the bird kidneys do not lend themselves to dissection for weighing, but they appeared to be proportionately reduced in size in the dwarfed embryos. Table I summarizes the results of the one-half and one-milligram series.

One-half mg series. Only the liver showed a significant reduction in weight, being reduced to approximately 86.5% of the control weight. The embryos and their membranes were similar to the controls.

One mg series. The entire embryo showed a growth inhibition following treatment with one milligram of cortisone. The liver was reduced to approximately 70% of its control

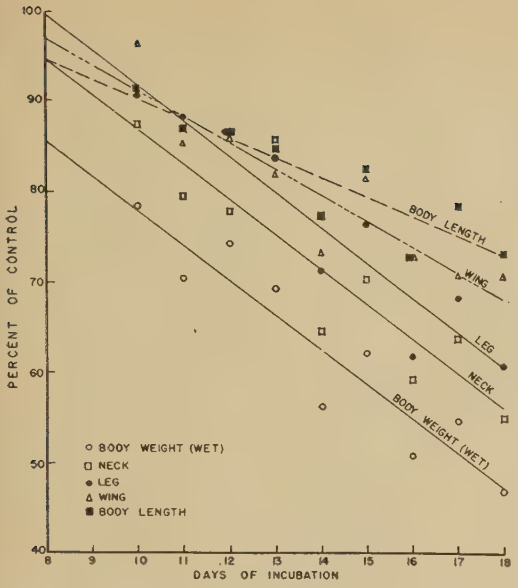

FIG. 1. Graph shows inhibition of growth of the embryo following injection at 8 days with 1 mg cortisone. Lines are extrapolated back to the day of injection. For wing, leg and body wt and body length $p < .001$, and for the neck $p < .01$.

proportional weight. The gonads—both ovaries and testes—and the eyes were relatively heavier. The ovaries of the treated embryos were about 250% of the control weight while the testes represented about 150% of the control weight. The eyes were approximately 142% of their controls.

In embryos showing the most marked cortisone-effect the amnion and chorion formed a tight-fitting double layered sac and the allantois and yolk sac were greatly inhibited. Such yolk sacs had thick convoluted walls which sat on the yolk like a skull cap. Although the yolk inside this cap-like sac was gelatinous, the rest of the yolk was liquid. Feather formation was inhibited over the entire body but most markedly on the head. The degree of feather inhibition is so variable that such an endpoint is not feasible for assaying cortisone activity. The inhibition of feather growth has been reported(3) and might be expected to occur, since it is known that cortisone produces atrophy of the skin and inhibition of hair growth in mammals(1).

The lengths of the body, neck, wings and legs were less in the treated than in control chicks. This is plotted in Fig. 1. The slope of the lines was determined by the method of least squares and the lines are extrapolated back to the eighth day when cortisone was administered. The greatest inhibition seems to be between 8 and 10 days for all parts except the legs.

The body wall of the treated embryos was generally translucent and many embryos were edematous. Comparison of wet and dry weights showed that the treated embryos retained water (Fig. 2). Control embryos began to show a decrease in water content at about 13 days of incubation, but the difference in water content between the treated and control embryos became statistically significant at 15 days of incubation.

Discussion. This study confirms the general results of Karnofsky *et al.*(3) that cortisone acetate inhibits growth and development of the chick embryo beyond 8 to 10 days of incubation. This inhibition is proportional, except that the liver is relatively smaller while the eyes and gonads are relatively larger.

Karnofsky(2) has given cortisone to day-old (40-45 g) chicks at a dose level of 5 mg per day for 10 days. By converting his average weights for several organs to milligrams of organ weight per 100 g of body weight, the following approximate percentages are obtained for immature cortisone-treated chicks: liver, 123% of the control weight; spleen, 76%; adrenals, 113%; heart, 123%. Probably not all of these values are significant. They do indicate a difference both in growth

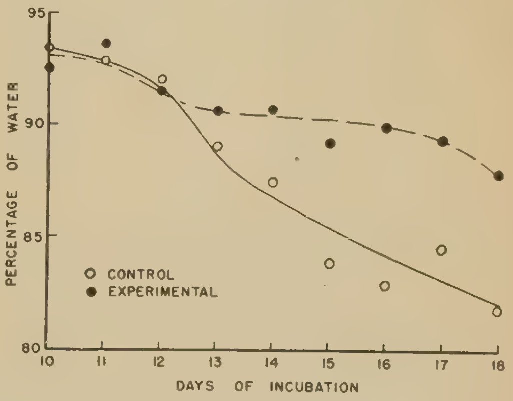

FIG. 2. Percentage of water in the entire embryo. The differences are significant on and after 15 days of incubation.

inhibition of embryonic and newly-hatched chicks and in response to various dosage levels.

Summary. 1. Chick embryos at 8 days of incubation were given one-half or one mg of cortisone acetate by injection onto the chorio-allantoic membrane. 2. The one-half mg dose resulted in a reduction of the liver to approximately 86% of the control weight. 3. In the one mg series, the liver was relatively smaller and the eyes and gonads were relatively larger on the basis of 100 g of body weight. In absolute values, the entire embryo and all organs were greatly reduced in size. 4. Measurements of body length, neck, wings and legs after treatment with one mg cortisone indicated the greatest degree of growth inhibition during 8 to 10 days of incubation for all except the legs. 5. A comparison of wet and dry weights from day 10 through day 18 of incubation showed that cortisone-treated embryos retained water. This retention becomes statistically significant at 15 days of incubation.

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Fibroma Arising from Feather Follicle of Duck Following Local Application of Methylcholanthrene to Skin.* (20256)

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The occurrence of papillomata, squamous cell carcinomata and hemangiomata in the skin of the duck following the local application of methylcholanthrene has been reported (1). In similarly treated ducks a fibroma has been observed to arise from the feather follicles. The pathogenesis and pathologic characteristics of this latter tumor are reported.

Methods and results. The technic used in the local application of a 0.25% acetone solution of methylcholanthrene to the skin of White Pekin ducks has been described(1). Ducks varying in age from 10 to 90 days were used. The birds were observed for a period of 6 months. The feathers were plucked frequently both during and after the local application of methylcholanthrene. This carcinogen was applied daily for 30 days by dropping 0.5 to 2.0 cc of the solution on the skin beneath the right wing and the adjacent portion of the body wall. The neoplasms in some of the ducks were removed for pathological study. Portions of skin showing developing feathers were also removed for histological study. A normal feather follicle is shown in Fig. 1A.

The feather shaft normally is composed of a loose fibrous tissue stroma with few blood vessels. The periphery of the feather as it emerges from the skin is covered by a thin layer of squamous epithelium. In ducks where the feathers are plucked frequently, the new feathers may fail to mature and may appear as a small projection 2-6 mm above the surface of the skin as illustrated in Fig. 2D. These abnormal feathers may become infected, hemorrhagic, gradually become dry, black structures that atrophy and then desquamate; others only atrophy and desquamate. Fig. 1B shows the macroscopic features of a tumor arising from such a follicle. Note the small feather growing from this tumor. Within 48 hours following the time this lesion was photographed, it spontaneously disappeared.

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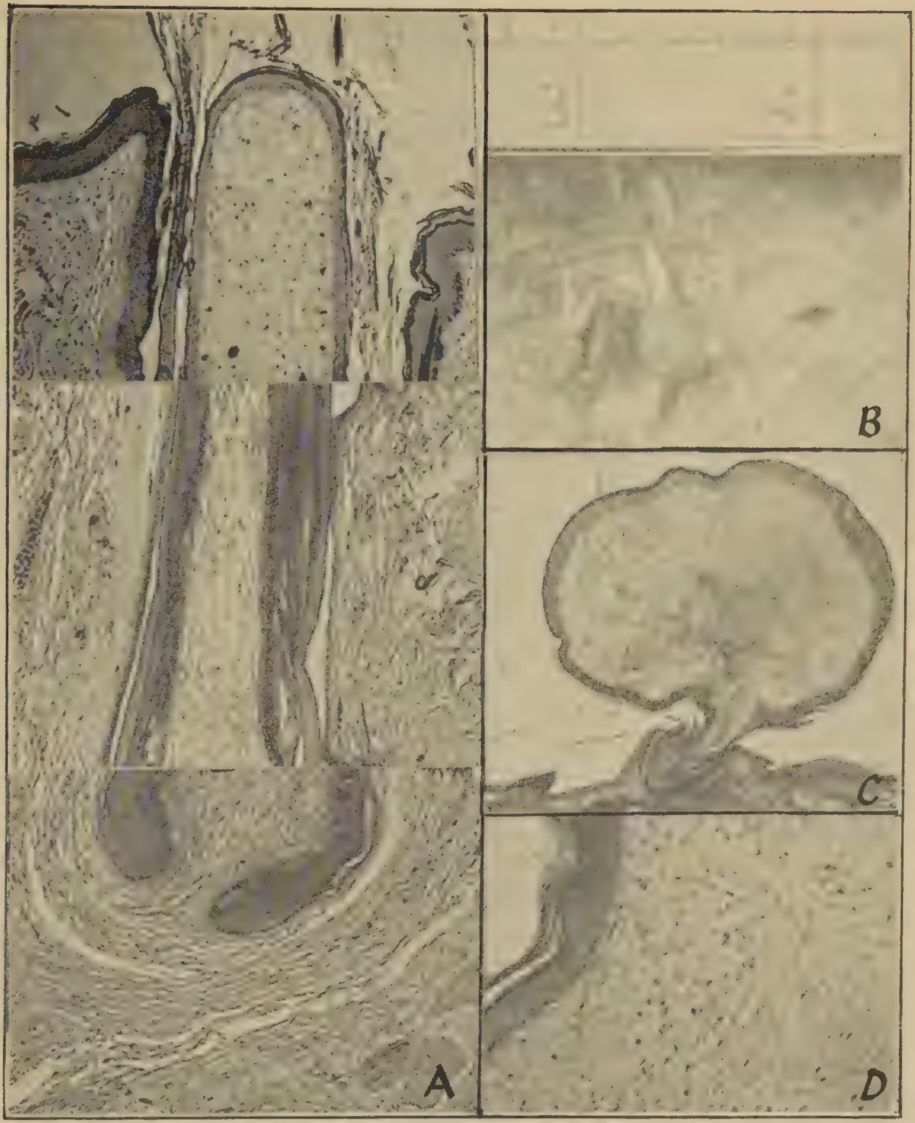

FIG. 1. A. A normal feather. Note the loose fibrous tissue stroma and the layer of squamous cells surrounding this developing feather. B. Duck 416. A fibroma 4 mm in diameter growing from a feather follicle. C. Duck 418. A fibroma growing from a feather follicle. H & E stain × 16 mm. D. Duck 407. Usually these fibromata show relatively few fibroblasts with squamous cells covering the periphery of the tumor. H & E stain × 150.

The narrow base of such tumors may account for their spontaneous disappearance because of the poor blood supply. This feature also would contribute to the fact that these neoplasms are usually small. The largest tumor observed in this study was 1.0 cm. Six ducks have shown this oval shaped tumor attached by a narrow pedicle. Many fibroma-like tumors with a large amount of neoplastic tissue continuous with the corium have been observed in these methylcholanthrene-treated ducks. It is difficult to determine the exact site of origin of the latter group of fibromata.

Histologically these tumors are composed of fibroblasts, a few small blood vessels, and as the tumor ages groups of lymphocytes make their appearance within the neoplastic tissue. The periphery of the tumors

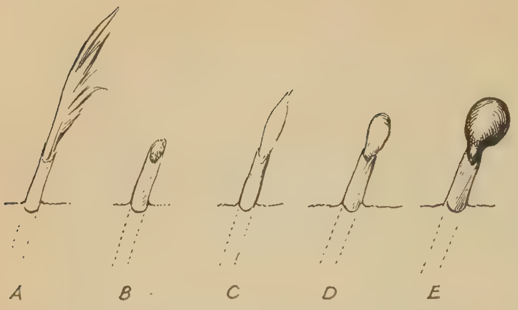

FIG. 2. Diagram of a feather (a) normal; (b) feather has been plucked leaving the shaft; (c) feather regrowing normally; (d) degenerating feather observed after frequent plucking; (e) tumor developing from a feather follicle.

is covered by a narrow layer of squamous epithelial cells (Fig. 1C and D).

Discussion. The skin of ducks treated with methylcholanthrene in acetone shows a wide variety of both histologically malignant and benign neoplasms. All tumors observed so far in more than 100 ducks have spontaneously disappeared except for those removed for histological study. The characteristics of these regressing tumors will be reported subsequently. The occurrence of a fibroma arising from the feather follicle is infrequent when compared with the occurrence of papillomata and hemangiomata in the skin of the duck. Many fibromata do occur in the skin and subcutaneous tissue of ducks treated with methylcholanthrene; however, their pathogenesis is more difficult to determine than the one arising from the feather follicle.

Ducks of similar age with feathers plucked and unplucked treated with acetone and normal ducks with their feathers plucked and unplucked have been observed for 6 months. No tumors have occurred. Degenerative changes, however, have been found in the feather follicles such as described above.

The duck has proven to be a satisfactory experimental host for the production of tumors with methylcholanthrene. The frequent occurrence of papillomata, squamous cell carcinomata and hemangiomata and the spontaneous regression of all neoplasms in the duck present a challenge in carcinogenesis.

Summary. A fibroma is described as arising from the feather follicle of White Pekin ducks following multiple local applications of methylcholanthrene to the skin.

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Surface Fixation as a Practical Method for Diagnosis of Brucellosis in Man and Animals. (20257)

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In spite of the considerable work developing and improving methods for the diagnosis of Brucellosis we still lack procedures which by themselves would detect all cases of human and animal diseases. It has been our experience that the more tests that are used the better the chance of obtaining an accurate diagnosis. It is for this reason that we have added to our routine, a new test, the results of which we consider of interest to report. In a previous paper we described a method for the detection of antigen antibody reactions which, so far, has been found applicable only to Brucella and its antibody(1). For practical purposes it was considered desirable to simplify the test, using material of uniform sensitivity and if possible standardized to produce reactions comparable in intensity to the results of tube agglutination tests. Considerable simplification of the test has been achieved by using antigens suspended in sugar cane syrup which may be printed on filter paper and which remain sensitive for several months. This change in the physical condition of the antigen allows for titration of its sensitivity to Brucella antibody in order to obtain results similar in significance to parallel tube agglutination tests.

In the present note we wish to present additional details concerning the preparation of the antigen, methods of standardization of the test and the results on human and bovine serum. In all cases the tests have been compared with tube agglutination tests and in the case of human patients with blood culture.

Preparation and standardization of the antigen. The method of staining the antigen has been already described(1). By fractional centrifugation, particles of precipitated haematoxylin are removed and then the suspension of stained *Br. abortus* ("S" form) is centrifuged and the sediment resuspended and centrifuged again to remove foreign material. The packed cells are transferred to a mortar and emulsified with freshly prepared sugar cane syrup. This must be prepared with refined sugar and heating to saturation. About 4 volumes of syrup are necessary to emulsify one volume of the sediment. The antigen is standardized to produce reactions of proper intensity when treated with Brucella-antiserum of known agglutinin titre. Since it is known that reactions performed by the tube dilution method are significant only when above certain titre, it has been our aim to produce antigens sensitive to titres above 1:100. For this purpose the antigen and its sugar content are adjusted after testing several dilutions of the stock antigen either with saline alone or with a mixture of saline and syrup. Various preparations are printed on filter paper (Eaton Dikeman No. 609) cut in pieces of 12 by 6 inches. The prints are made by means of a circular rubber stamp or glass rod 3 mm in diameter at a distance of 1 inch from one of the large ends of the paper. The dried spots are treated with the test serum and also with a negative serum control as indicated below. For practical purposes it is advisable to select the antigen which produces from 50 to 75% fixation with a serum of 1:160 titre. The selected sample is used as a guide in the preparation of the antigen. This is printed on the paper as previously indicated and is ready for use the next day. The printed material should be kept in a dry place at room temperature.

Test. For a single test, paper containing 3 spots is cut transversally from the leaf. Over the middle spot a droplet of unknown serum is placed with a wire loop 3 mm diameter. On each side a positive and negative control are placed with similar loops. Before the serum dries, the paper is hung with its end in a layer of isotonic NaCl solution taking care that the spots remain about half an inch over the surface of the fluid. From 20 to 25 minutes may be required for the absorbed fluid to reach the upper part of the paper. *Readings.* The negative control will show a trail of antigen starting from the spot, to nearly the upper part of the paper (about 4 inches). The positive control will remain fixed. The titre of the unknown serum may be estimated according to its behaviour in relation to the controls. Accordingly, the intensity of the reaction, when positive, may be graded either by the customary 1 to 4 plus or by approximate percentages of fixation.

Results. Table I shows the results of 906 tests performed with serum obtained from 590 patients suffering from brucellosis in whom the diagnosis was proven by the isolation of Brucella from the blood or by an agglutination test of high titre. The cases are classified according to the results of the agglutinin titre as obtained by the tube dilution method. The results of the paper test are classified according to percentages of fixation. As controls we include 221 tests performed on patients suffering from various acute and chronic infections not including brucellosis. About 10% of these cases had agglutinin titres for Brucella ranging from 1:20 to 1:80. There is another group of 100 tests performed with normal human, guinea-pig and rabbit sera.

From this table several observations may be made: 1) No negative "surface fixation" tests were shown in proven cases of active brucellosis. 2) Low titres of agglutinin are usually concomitant with weak "surface fixation" tests. 3) The intensity of the "surface fixation" test increases with increasing agglutinin titres.

Considerable discrepancies, however, have been observed with sera of low agglutinin titres. Nearly 3% of all tests showed a negative agglutination test. The diagnosis, how-

TABLE I. "Surface Fixation" Compared with Tube Agglutination in Human Brucellosis Proven by Blood Culture or High Agglutinin Titres.

Tube agglut. titre	No. of cases	Results of surface fixation							
		25%		50%		75%		100%	
		No.	%	No.	%	No.	%	No.	%
Negative*	27	16	59.2	7	25.9	4	14.8	0	0
1:20 *	15	12	80.0	1	6.6	0	0	2	13.3
1:40 *	21	13	62.0	5	23.8	2	9.5	1	4.7
1:80 *	77	30	38.9	25	32.5	17	22.1	5	6.5
1:160	113	10	8.8	41	36.3	40	35.4	22	19.5
1:320	194	10	5.0	51	26.6	93	47.9	40	20.5
1:640 and over	459	6	1.3	35	7.7	181	39.4	237	51.6
Total	906 cases of brucellosis.								

Control groups: 1*—221 patients suffering from various infections not including brucellosis, some with agglutinin titres of 1:20 to 1:80. Negative "surface fixation"

2*—100 normal human, guinea pig and rabbit sera. Negative "surface fixation"

* These cases had simultaneous or recent positive blood cultures.

ever, was based on simultaneous or recent isolation of Brucella from the blood as it was the case with the following 3 groups of patients giving titres from 1:20 to 1:80. Since titres below 1:100 have no diagnostic value by themselves, the percentages of cases of active brucellosis which may not be detected by a single tube agglutination test increases to 12%. Since in all these cases antibody may be detected by "surface fixation", such failure to be shown by tube agglutination may depend on the possible interference by blocking phenomena(2). Otherwise, the observation of patients for long periods after the acute phase of brucellosis has shown in general a parallel decrease in intensity of both agglutinin titre and "surface fixation" reaction.

TABLE II. Sample of Survey with "Surface Fixation" and Tube Agglutination on Bovines from the Same Herd.

Serial No.	Agglutinin titre	% of fixation
75	20	0
76	640	100
77	160	50
78	10	0
79	40	0
80	10	0
97	1280	100
98	320	75
99	40	0
100	80	50
101	20	25
102	80	50

TABLE III. "Surface Fixation" and Agglutination Test with Sera from 716 Bovines from Several Herds.

No. of animals tested	Agglutinin titre	Results according to % of fixation				
		0	25	50	75	100
318	Negative	318	—	—	—	—
136	1:10	134	2	—	—	—
95	1:20	86	8	1	—	—
61	1:40	45	13	3	—	—
25	1:80	11	8	5	1	—
81	>100	2	8	15	16	40
716		596	39	24	17	40

A comparative study of "surface fixation" and tube agglutination test performed with bovine serum has given similar results, although discrepancies cannot be discussed on the same basis as in human patients because of the impossibility to establish the diagnosis in random cases by means of blood culture. About 1,500 comparative tests have been performed and the results summarized in Tables II and III.

Because of the way in which the paper test has been standardized one would not expect clear cut positive reactions with sera of titres below 1:100. However, in Table II it may be seen that in some cases fixation as high as 50% may be shown by sera of titres of 1:80. The interpretation of such results is difficult because of reasons previously given.

It is of interest to observe Table III in which a group of 716 bovines were tested by the 2 methods. The correlation between negative "surface fixation" and negative tube agglutination test was correct, but nearly 13% of the sera with agglutination tests from 1:10

to 1:80 produced positive "surface fixation" tests of different intensity. These discrepancies may be due to inhibitory factors in the agglutination test which have no influence on the paper test.

It may be noticed that stronger "surface fixation" tests are to be found in sera of titres above 1:100. On the other hand, 2 out of 81 cases with significant tube agglutination tests failed to produce "surface fixation" reaction. The only possible explanation of the latter discrepancy may be related to the rather low sensitivity of the paper to sera with agglutinin titres close to 1:100.

Summary. Attempts have been made to standardize the "surface fixation" test for the diagnosis of brucellosis and at the same time make it a practical rapid and dependable procedure. So far, it has been found to possess remarkable specificity in human brucellosis as judged by comparison with blood cultures. This test may be applicable to the detection of bovine reactors.

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Biosynthesis of Vitamin C: A New Precursor. (20258)

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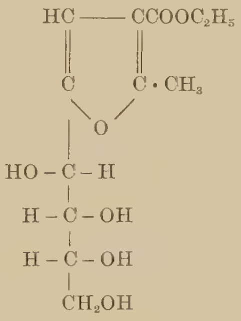
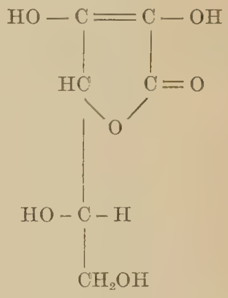
Much work has been done on the biosynthesis of vit. C, but the precise mechanism is still to be revealed. Ray(1), Smythe, and King(2) and several others have indicated that hexose sugars and sugar acids are in some way concerned in the biosynthesis, and according to Mentzer and Urbain(3) lipids also can serve as the precursor of ascorbic acid in the body of the rat.

Acetoacetate, an intermediary metabolite of lipids found by Nath and associates(4,5) to cause an immediate rise in the blood glucose and decrease in glucose tolerance, has recently been reported(6) to cause a sudden increase in vit. C of blood of rat. Because glucose as well as acetoacetate, in small doses, accelerated biosynthesis of vit. C, it was thought desirable to see the combined effect of the two in germinating mung bean (*Phaseolus mungo*), as well as in the body of the rat. It has recently been briefly reported(7) that though acetoacetate alone has a retarding effect on biosynthesis of vit. C in the germinating mung bean, acetoacetate and glucose added to the medium in equimolecular proportion cause a gradual rise of vit. C. The condensation product of the two(8) which Gonzales(9) showed to be 2-tetrahydroxy butyl 5-methyl 4-carbethoxy furan (I) sharply raised the vit. C (II) level of the germinating mung bean from the start of the experiment.


(I)


(II)

Experimental. (A) *Biosynthesis in germinating mung bean.* Glucose and acetoacetate (Na salt) separately or together and the condensation product of the two were added to the medium for germination of mung bean. Seeds were first soaked in glass-distilled water for about 3 hours, then immersed in aqueous media containing these different substances, at pH 7.4. For each experiment 500 seeds of almost equal weight were selected. Potassium meta bi-sulphite (300 P.P.m.) was added to all media as recommended by Kendrick and Downer(10). One hundred seeds were taken when required, the testa removed and ex-

TABLE I. Effect of Using Equimolecular Proportions of Different Substances on the Production of Ascorbic Acid During Process of Germination of Legumes.

Substances used in germinating medium	Vit. C (mg)/100 germinated seeds and mean deviation after germination 24 hr	48 hr	72 hr	96 hr
None	.91 ± .19	1.81 ± .16	2.36 ± .18	2.08 ± .12
Glucose (a)*	1.05 ± .15	1.94 ± .14	2.48 ± .18	2.13 ± .09
Sodium acetoacetate (b)*	.81 ± .04	.61 ± .03	.50 ± .02	.38 ± .09
Glucose + sodium acetoacetate*	.85 ± .10	1.92 ± .06	2.24 ± .17	2.56 ± .19
Condensation product of (a)* and (b)*	1.31 ± .16	2.67 ± .12	3.64 ± .14	4.07 ± .25

* Added in equimolar amounts; glucose being 100 mg.

TABLE II. Effect of Concentration of Condensation Product on the Vitamin C Synthesis.

Substances in germinating medium	Amt of substance (mg/100 seeds)	Vit. C, mg/100 germinated seeds 24 hr	48 hr	72 hr	96 hr
Glucose	100	.79	1.85	2.40	2.16
Condensation product	5	1.28	2.00	2.79	2.79
—	25	1.30	2.60	3.00	4.12
—	100	1.37	3.00	4.08	4.15
—	250	1.42	2.92	4.05	4.15

tracted thrice with a mixture of metaphosphoric acid (3%) and acetic acid (8%) and ascorbic acid estimated according to titrimetric method of Bessey(11). Because this method may lead to erroneous results owing to interference from other reducing compounds, the experiment was repeated and ascorbic acid estimated colorimetrically according to the method of Roe and Kuether (12). In all the above processes of germination the seeds were first washed with .1% mercuric chloride solution, then with sterile water and finally kept in sterile petri dishes with the required amounts of sterile distilled water for ideal germination. The results obtained with the Roe and Kuether's method are shown in Table I.

Some experiments were then done to show whether the condensation product itself inhibits the breakdown of the ascorbic acid. Equal amounts of extracts of the germinated beans were placed in 2 sets of flasks, one set containing the condensation product. Known quantities of ascorbic acid were added to all the flasks and the amounts of ascorbic acid in both the sets were determined at regular intervals. The same degree of breakdown was noticed in both the sets. After it was seen that the concentration of vit. C in the germinated seeds is about 100% higher after 72 hours in the dishes containing the condensation product of glucose and acetoacetate (152 mg per 100 seeds), it was thought desirable to study the effect of the concentration of the product on the amount of vit. C formed. The solubility of the condensation product (I) is about 100 mg in 10 cc of water at about 27°C (room temp.).

For the higher concentration of the condensation product, a suspension of the same was made in water because of its poor solubility. The results are shown in Table II.

Table III shows that in a mixture of glucose and acetoacetate a high proportion of the latter depresses the biosynthesis of vit. C.

In order to see if there was any indication of some chemical reaction between glucose and acetoacetate at pH 7.4 (37°C), experiments were carried out with known amounts of glucose with and without acetoacetate. A substantial decrease in glucose concentration

TABLE III. Effect of Glucose with Low and High Concentration of Sodium Acetoacetate on Vit. C Biosynthesis in Germinating Seeds.

Substances in germinating medium	Amt of substance (mg/100 seeds)	Vit. C, mg/100 germinating seeds 24 hr	48 hr	72 hr
Water		.96	1.84	2.47
Glucose	100	.80	2.16	2.67
Glucose & Na acetoacetate	10 10	.99	2.35	2.88
Glucose & Na acetoacetate	10 150	.52	.78	1.99

TABLE IV. Biosynthesis of Vit. C in Rats Caused by Intramusc. Inj. of Glucose, Acetoacetate Followed by Its Equimolecular Amount of Glucose, Condensation Product of Glucose and Ethylacetate and Acetoacetate Respectively (3 Rats in Each Series).

Substances inj. (mg/100 g body wt)	Avg values of vit. C/100 cc plasma (mg) 0 hr	1 hr	4 hr
Acetoacetate, 100	1.40 ± .03	1.80 ± .04	.70 ± .02
Glucose, 145.2	1.41 ± .02	1.70 ± .08	1.41 ± .02
Acetoacetate, 100, immediately followed by glucose, 145.2	1.42 ± .02	1.98 ± .18	1.42 ± .05
Condensation product of glucose & ethylacetoacetate, 25 in solution	1.42 ± .02	2.5 ± .05	1.42 ± .02
Ascorbic acid, 16 (equivalent to 25 condensation product)	1.41 ± .02	2.9 ± .18	1.41 ± .02

was observed after 72 hours of incubation in presence of acetoacetate. Experiments on the effect of acetoacetate in germinating medium on utilization of added glucose also indicated great loss of glucose after germination for 24 hours in presence of acetoacetate, provided its quantity did not exceed the equimolecular amount of glucose.

(B) *Biosynthesis of ascorbic acid in the animal system.* It was thought necessary to see how glucose and acetoacetate can influence the synthesis of vit. C in the body of the rat, which is known to synthesize the vitamin. Rats weighing about 180 g were used for the experiment, and the different substances were injected into them intramuscularly. Blood was collected from rats by clipping the tails. 0.7 cc of blood was collected for each estimation, before and after injections, and plasma was obtained after centrifuging. To 0.3 cc of the plasma 2.7 cc of 5% metaphosphoric acid was added for deproteinization. The clear extract obtained after centrifuging was used for the determination of ascorbic acid according to the method of Mindlin and Butler(13) by the use of the photoelectric colorimeter. The results are shown in Table IV.

Discussion. Szeki and Laszlow(14) succeeded in oxidizing the condensation product (I) to 5-formyl furan 3-carboxylic ester, furan 3-5-dicarboxylic acid and furan 1-3-5-tricarboxylic acid respectively. Again, it is quite interesting to note that the work of Muller and Varga(15) on the oxidation of the condensation product (I) with excess of $Pb(oAc)_4$ has definitely revealed a cleavage between the 4-5 C atoms of the original carbon chain of the glucose molecule. Thus the transformation of the condensation product (I) into vit. C (II) may be pictured through the combined process of oxidation and hydration which might be taking place during the course of germination.

Further, the possibility of the conversion of the condensation product to any substance or substances other than ascorbic acid, having the property of reducing the dye, can also be eliminated through the recent findings of Nath and Sahu(16) who, as a result of hydrolytic and oxidative effects of HCl and alkaline H_2O_2 respectively on the condensation product, obtained a number of substances, none of which can reduce the dye. That there is a chemical reaction taking place between glucose and acetoacetate is also indicated by the formation of a yellow color in the reaction mixture. This color formation, observed after 24 hours incubation, deepens in intensity if the period of incubation is further increased to 48, 72, or 96 hours, thus showing that there is more and more of a reaction between the two, glucose and acetoacetate. That the color formation is only due to a reaction between glucose and acetoacetate and not between glucose and acetone, which is formed as a result of hydrolysis of Na acetoacetate, was nicely indicated by a control experiment wherein glucose was incubated with acetone under identical conditions. Further, the findings of Illari(17) are also in accord with our view of a reaction between glucose and acetoacetate. The data indicate that the biosynthesis of ascorbic acid is the result of a reaction between glucose and acetoacetate which in all probability appears to be an enzymatic one.

It is possible that the metabolic relationship between glucose and acetoacetate, which has just been reported by Nath and Sahu(18) to exist in the living body, assumes a much greater significance and importance in both animals and plants.

That this condensation product of glucose and acetoacetate acts as a precursor to ascorbic acid has also been substantiated by *in vivo* studies on rats. Higher values of the vit. C in the plasma of rats have been noticed (Table IV) after one hour of injection of acetoacetate following that of glucose or of the condensation product of the two given in water solution. Further, these data also show the beneficial role of glucose towards the toxic effect of acetoacetate and supports the view of a metabolic relationship between these two compounds(18).

Summary. 1. Though glucose has a slight effect in the process of biosynthesis of ascorbic acid in the germinating mung beans and acetoacetate alone has rather a destructive effect, the product formed by the condensation of the two (2-tetra hydroxy butyl 5-methyl 4-carbethoxy furan) has been found to accelerate the process greatly. 2. Glucose and acetoacetate when added in equimolecular amounts to the germinating beans have also been shown to cause greater biosynthesis of ascorbic acid. 3. The condensation product of glucose and acetoacetate has no inhibitory effect on the loss of ascorbic acid and the probability of this compound, which is also structurally similar to ascorbic acid, acting as a precursor of ascorbic acid has been suggested. 4. Injection of the condensation product of glucose and acetoacetate or of one following the other has been found to raise the ascorbic acid level in the plasma of rats. 5. Glucose has been found to prevent the harmful effect of acetoacetate with respect to depletion of ascorbic acid *in vivo*. 6. Pathways of glucose metabolism not previously recognized have been suggested.

Addendum. After this paper was communicated for publication, a paper on the origin of l-ascorbic acid in the albino rats has appeared from the laboratory of King (19), which gives confirmatory evidence of glucose taking part in the biosynthesis of ascorbic acid.

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Effect of Aureomycin on Utilization of Vitamin A in the Growing Male Albino Rat. (20259)

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Recent investigations by Lih and Baumann (1) and by Sauberlich(2) have shown that antibiotics exert a "sparing effect" on the requirements of vitamins of the "B" complex in the rat. The nature of this "sparing" has not been elucidated, but is generally thought to be due either to an increased synthesis of B vitamins by micro-organisms of the intestinal tract, or to an increased efficiency of absorption of the vitamin from the tract. Burgess *et al.*(3) and also Coates *et al.*(3a) observed in the case of the chick receiving adequate vit. A or precursors plus Na-penicillin G at a level of 30 mg/kg of diet, in addition to significantly increased body weight, a statistically significant ($p = .01$) increase in both total liver vit. A and vit. A per gram of fresh liver.

It was thought that investigations of the effect of an antibiotic (aureomycin) on the vit. A requirement of the growing male albino rat, using growth as a criteria of dietary vit. A adequacy, would be of interest. In addition to ascertaining whether an antibiotic increases liver stores of vit. A in the rat as in the chick, data on kidney stores were also obtained since Johnson and Baumann(4) and Eden and Moore(5) report that on low intakes of vit. A or carotenoids kidney storage of vit. A exceeds liver storage in the rat. It was also thought that this experiment might elucidate somewhat the function of antibiotics. If antibiotics act in growth stimulation by aiding in absorption of a vitamin, partial sparing of the vit. A requirement would be expected, whereas if such function be one of aiding in intestinal synthesis no sparing of vit. A would be anticipated.

Experimental. In an experiment replicated twice male rats were randomly assigned to 6 lots of 5 animals each. They were kept in individual screen bottom cages and given food and water *ad libitum*. The animals were weighed at weekly intervals, while weekly food consumption was determined by weighing back left-over food on the same day the animals were weighed. Both experiments lasted 7 weeks and upon termination vit. A assays were made on the liver and kidneys of each rat in the second replicate by the method of Gallup and Hoefer(6). The basal diet consisted of vitamin-free casein 22%, salt mix 446(7) 4%, cerelose 68.5%, cotton seed oil 5%, complete B vitamin mix* 0.1%, choline (25%) dry mix 0.4%, plus delsterol added to the complete diet at 0.05%. The natural ester form of vitamin A was administered to each rat daily in the form of 0.1 ml of a petroleum ether (skellysolve B) solution of vit. A and D concentrate oil† added to 2 g of the diet in a manner identical to that employed by Koehn(8). The petroleum ether solution contained 1.0 mg α-tocopherol acetate per 0.1 ml of solution. Aureomycin was the antibiotic used. Table I summarizes the treatments given the respective lots. The levels of vit. A ester were chosen so that lots III and IV would be receiving a sub-optimal level for maximum rate of growth, while lots V and VI would be receiving a level above the optimal [Koehn(8)].

TABLE I. Experimental Feeding Plan.

	Additions to vit. A—low basal diet	
Lot No.	Vitamin A, γ/rat/day	Aureomycin, mg/kg diet
1	0	0
2	0	100
3	.75	0
4	.75	100
5	7.5	0
6	7.5	100

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* Vit. premix:		
	Thiamine HCl	500 mg
	Riboflavin	600 ”
	Pyridoxine HCl	300 ”
	Ca. pantothenate	3 g
	Nicotinic acid	6 ”
	Folic acid	100 mg
	Vit. K	100 ”
	Vit. B_{12}	.5 ”
	Biotin	10.0 ”
	Cerelose to make	100 g total

† Distillation Products Industries, Rochester, N. Y.

TABLE II. Average Total Weight Gains and Food Consumption at 49 Days.

Group	A (vit. A-free)		B (0.75 γ vit. A/day)		C (7.5 γ vit. A/day)	
Lot No.	1*	2† (aureomycin)	3	4 (aureomycin)	5	6 (aureomycin)
Wt gain, g	75.6‡	66.9	198.5	180.2	251.6	252.9
Food consumption, g	258 ‡	209	485	487	601	639

* Values attained at time of death (survival time 42.4 ± 3.5 days).
† " " " " " " (" " 29.6 ± 1.8 days).

‡ Significant at $P < 0.05$ where

$$t = \left[\frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\dfrac{\Sigma x_1^2 + \Sigma x_2^1}{(N_1-1) + (N_2-1)}}} \times \sqrt{\frac{N_1 N_2}{N_1 + N_2}} \right]$$

where X = mean value, x = deviation from mean, N = No. of observations.

TABLE III. Vitamin A Content of Liver and Kidneys of Rats from Second Replicate.

Group	A		B		C*	
Lot	1	2 (aureomycin)	3	4 (aureomycin)	5	6 (aureomycin)
Total vit. A/liver, γ	0	0	0-trace	0-trace	75.9 ± 7.48	79.6 ±15.22
Vit. A, γ/g fresh liver	0	0	"	"	7.8 ± 1.22	6.5 ± 1.14
Total vit. A/pair kidneys, γ	0	0	"	"	3.7 ± .54†	1.5§± .37
Vit. A, γ/g fresh kidney	0	0	"	"	2.2 ± .25‡	.9§± .21

* All values are expressed as mean ± stand. error.
† Significant at P = .05. ‡ Significant at P = .01.
§ 4 animals only. All other figures refer to 5 animals.

Results and discussion. Weight gain and food consumption data for the complete experiment are summarized in Table II. In Table III are given the vit. A contents of the liver and kidneys of the rats in the second replicate. Differences between groups A, B and C (Tables II and III) have been tested for statistical significance by comparing the highest mean value in section A (*i.e.*, 1 or 2) with the lowest in section B (3 or 4) etc. All differences were found to be significant at the 0.001 level except food consumption of groups B and C which only reach the 0.01 level.

In the case of both lots I and II the typical syndrome of avitaminosis A was observed, but it was noted that the symptoms appeared earlier in the aureomycin treated animals and seemed to be more severe. This observation is given increased significance when survival times for the lots concerned are compared. Emslie(9) in a personal communication with one of us (B.C.J.), referring to work previously cited(3), indicated that in the chick a vit. A deficiency is produced more rapidly on a vit. A deficient diet plus an antibiotic than on a vit. A deficient diet alone. It appears from Table II that the animals of lot I made greater gains than the animals of lot II due to the greater food intake. Animals receiving sub-optimal or above-optimal levels of vit. A do not demonstrate the effects of increased food consumption or increased weight gains when aureomycin is added to the diet.

The vit. A assay results of livers and kidneys are in reasonably good agreement with the published data of Moore and Sharman (10).

In both lots V and VI liver storage far exceeded kidney storage both on the basis of total content and concentration; comparison here with the previously cited work is not possible because the duration of supplementation is probably critical. It is interesting to note at this point that kidney storage of vit. A in lot 6 was significantly lower than that of lot 5 both as to total amount present and as to concentration in the fresh tissue. An elucidation of this finding must await further investigation.

No vit. A could be detected on analysis of individual kidneys and livers of rats of lots III and IV as evidenced by the lack of blue coloration with $SbCl_3$ reagent. Petroleum ether extracts of livers and of kidneys from these lots were pooled in order to obtain a greater concentration of vitamin per colorimeter tube. These showed only a very slight blue coloration. From consideration of the data of Moore and Sharman(10) it is evident that very small amounts of vit. A should be present in the tissues concerned at 0.75 γ per day level of supplementation.

It would appear, on the basis of these data, that aureomycin does not function in the absorption of vit. A from the tract. Whether this is true also for the B vitamins requires additional experimental work.

Summary. 1. Growing male albino rats were fed vit. A ester at levels of 0, 0.75, and 7.5 γ/rat/day. Comparisons were made at these levels of vit. A intake with and without aureomycin added to the diet at the rate of 100 mg/kg. 2. Statistically significant differences were found in weight gains and food intakes between groups receiving different levels of vit. A. 3. Statistically significant differences were not found in weight gains and food intakes between groups receiving antibiotic and their controls at the various levels of intake of vit. A, except on vit. A-free diets. Here weight gains and food intake of the control animals were significantly greater than for animals receiving antibiotics. 4. At a level of intake of vit. A of 7.5 γ/day no significant difference of liver storage was found upon aureomycin supplementation. Kidney storage of aureomycin supplemented animals was significantly less than that of control animals. Under conditions of this experiment at the 7.5 γ/day level of supplementation of vit. A significantly greater storage of vit. A occurred in the liver than in the kidneys. 5. No effect of sparing of vit. A by aureomycin was demonstrated, but on vit. A-free diets aureomycin appeared to enhance the deficiency syndrome.

The aureomycin and folic acid used were generously supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y. through the courtesy of Dr. T. H. Jukes. The other B vitamins and vit. E were generously supplied by Merck and Co., Rahway, N. J. through the courtesy of Dr. Harold H. Draper.

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Acid Phosphatase in Human Female Genital Tract, A Histochemical and Biochemical Study.* (20260)

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In the course of histochemical studies of the normal and malignant human cervix uteri on the possible relationship of alkaline phosphatase activity at pH 7.4 and 9.0, with various phosphorylated derivatives, to glycogen deposition, surprisingly slight activity of the cervical epithelium was a consistent finding.

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Because of reports by Gomori(1,2) on marked phosphamidase activity in this tissue in the malignant state at pH 5.6, we studied histochemically the acid phosphatase activity. The enzyme activity was found in the normal cervical epithelium, in the vagina, endocervical glands, glands and stroma of the endometrium, mucosa of the fallopian tube, and, to a much lesser extent, the ovary. Supporting biochemical data were also obtained and so far, at least, no obvious contradictions between the two independent methods of study have appeared, though precise correlation and quantitation have not as yet been attempted.

Materials and methods. Tissues for histochemical studies were treated as previously described(3). Fragments of tissue, preferably less than 1 cc, were frozen in melting isopentane and stored at —30°C until sectioned. Sections were cut at 15 μ in a Linderstrom-Lang type cryostat and appressed to slides without benefit of adhesive. Incubation periods ranged from 10 minutes to 48 hours. The incubating medium was essentially as recommended by Gomori(4): 0.05 M CH_3COON_a; 0.01 M phosphorylated substrate; and 0.004 $(CH_3COO)_2Pb$. pH was adjusted with concentrated HCl. Using the principle of Friedenwald and coworkers(5), the solution was saturated with PO_4^{---} ions with the aid of concentrated Na_3PO_4. As already reported, 0.01 M NaF proved to be a very effective inhibitor of this reaction. *Biochemical studies,* using homogenate technics, were also performed on tissues of the female genital tract. Tissues, homogenized at concentrations of 1-5% in distilled H_2O, were used as enzyme source. Lower concentrations of tissue were necessitated by paucity of material in such cases as the vaginal and cervical epithelium and fallopian tube mucosa, which were obtained by scraping the luminal surface of these organs. The homogenizer was chilled in an ice-water bath during maceration and in storage prior to actual running of the test. Various buffer or buffer combinations and two substrates were employed in the biochemical studies with the homogenates. In the acid range (pH 2.2 to 8.0) a simple CH_3COON_a (0.028 M) buffer (adjusted with HCl) appeared superior to one with added veronal. In the alkaline range (9.0 to 10.6 or 11.0) veronal alone (0.028 M) appeared satisfactory. Disodium phenylphosphate (0.005 M) was used as a substrate besides sodium glycerophosphate (0.01 or 0.02 M), since it has been reported to be more readily hydrolyzed than the latter (*i.e.* glycerophosphate). As a rule the homogenate (1-5%) was introduced as 0.2 ml of tissue suspension into 2 ml of substrate-buffer mixture. The reaction was terminated by the addition of 0.5 ml of 20% $C\,Cl_3COOH$. Inorganic PO_4^{---} assays, as a measure of enzyme activity, were performed by the method of Fiske and Subbarow(6) on 1 ml aliquots of supernatant from centrifuged reaction mixtures. Controls consisted of tubes (a) lacking only substrate, (b) lacking only homogenate, (c) lacking both, and (d) to which TCA was added prior to incubation period.

Results. Biochemical. Except for the sample of endocervical tissue, the amount of alkaline phosphatase activity has been a fifth of the acid variety, or even less. In order to characterize more closely the acid phosphatase activity encountered, assays for phosphatase activity were run at various pH levels from 2.2 to 11.0, usually in steps of 1 pH unit, and in the presence of some selected inhibitors, which, from the work of Abul-Fadl and King(7) among others, could be expected to be informative. Because endometrium was available to us in greater quantities than any of the other tissues studied, most of these experiments were performed with this tissue. Rat liver and human prostate (benign adenoma) were assayed, using identical technics in independent and parallel runs for comparative purposes.

pH. The acid phosphatase activity of the female genital tract has a pH optimum around 5. The peak for this tissue, as for other tissues simultaneously investigated, does not appear sharp. Considerable activity has been encountered at pH 4, or even a little lower (3.4), and marked inorganic phosphate release obtains at as high as pH 7 or slightly above (Fig. 1).

Inhibitors. Like others, we have found that 0.001 M NaF gives 100% inhibition of this enzymic activity (Fig. 1). Tartrate (0.02 M)

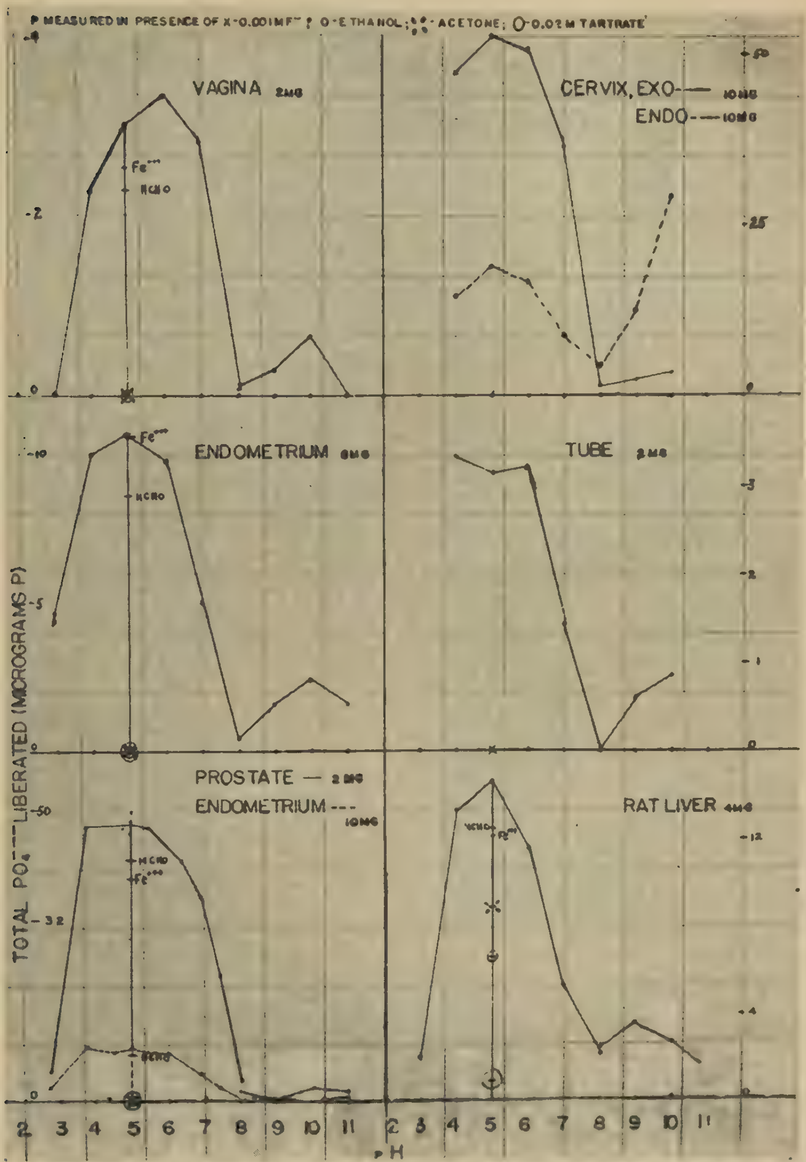

FIG. 1. Six curves, showing variation in total PO_4 liberated (expressed as μg P) by homogenized tissues from various linings of the female genital tract and from human prostate, and rat liver, at various pH levels in acid and alkaline range. The level of activity in presence of various inhibitors at pH 5 is indicated by horizontal marks on ordinates at pH 5. Amount of tissue (wet wt) per tube in each reaction mixture is indicated with each graph and ordinates vary with each graph as indicated. HCHO employed at 0.5%. Fe^{+++} employed at 0.001 M.

likewise has been found to give about this much inhibition. This has been found to depend to some extent on the substrate used. With disodium phenyl phosphate, tartrate inhibition in endometrium was not nearly as marked as with sodium glycerophosphate. Both alcohol and acetone, when incubated at 2/5 volumes concentration preliminary to substrate-buffer addition(7) were found to be very potent inhibitors of acid phosphatase activity in the female genital tract, but only about half as effective against rat liver phosphatase. This finding is in partial agreement with Abul-Fadl and King(7) who report

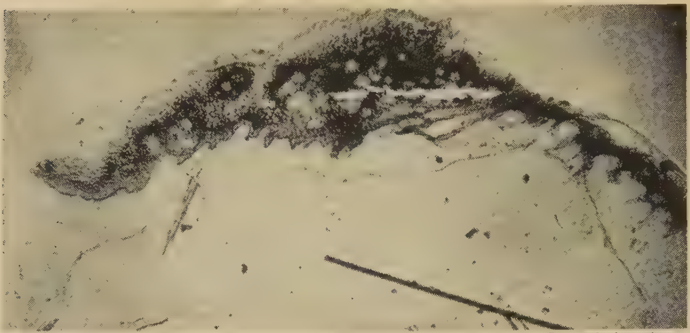

FIG. 2. Section of wall of vagina of human (× 25), showing marked activity in epithelium and stroma quite inert. Pointer is on stroma. Incubated with glycerophosphate for 2 hr at pH 5.

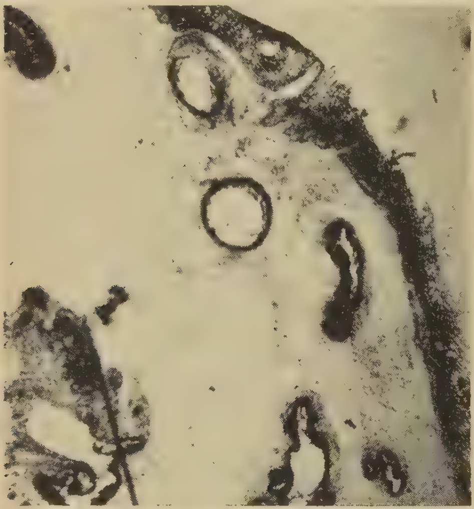

FIG. 3. Section of biopsy of human cervix uteri (× 25), showing marked activity in epithelium and very active endocervical glands. Tip of pointer is on basal layer of a short segment of epithelium. Incubated as above.

human liver less sensitive to alcohol and acetone inhibition than prostatic acid phosphatases. Fe^{+++} in the form of $Fe(NO_3)_3 \cdot 9H_2O$ (0.001 M) and 0.5% HCHO caused only slight inhibition of acid phosphatase activity.

Histochemical. There is a high level of acid phosphatase activity in the epithelial layers of the vagina and cervix (Fig. 2 and 3), but a much lower alkaline phosphatase activity. The same is true for the endometrium. All data given below refer to results obtained with glycerophosphate as substrate unless otherwise indicated.

Vagina. To date we have examined sections of the anterior and posterior wall of the vagina from one patient (Fig. 2). The basal layer of the epithelium does not stand out from the rest of the epithelium as much as it does in the exo-cervix. As in the latter, the zone of greatest activity lies 3 to several cell layers removed from the stroma, and toward the lumen of the organ, activity gradually becomes almost nil. In this case there seems to be an appreciable amount of nuclear activity, relatively more so than in the cervix.

Cervix. The epithelium of the pars vaginalis of the cervix reacts like the epithelium of the vagina. In general it is much more reactive than the stroma with the notable exception of the endocervical glands, which are intensely reactive (Fig. 3). The reaction in the epithelium is primarily cytoplasmic, though slight to rather strong nuclear activity has been noted on occasion. The reaction is by no means of uniform intensity across the thickness of the epithelium. There is an accentuation of deposition in the basal epithelial layer as compared to the stroma, and to a lesser extent as compared to the parabasal layers (Fig. 3). A zone of maximum phosphatase activity occurs at a region 2 to several cell layers removed from the basal layer. This zone of maximum activity does not extend entirely to the luminal border, the intensity of deposition as a rule diminishes gradually to about nothing as this border is approached, or even before the peripheral cell layer is reached. In this region of intense activity the deposition around the cell periphery is quite pronounced, particularly since the central area of these squamous cells has taken on a void-like appearance with the dissolution of contained glycogen. The epithelial cells of the endo-

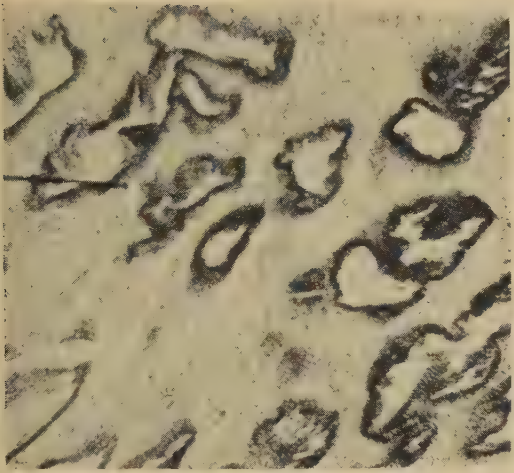

FIG. 4. Section of endometrium of human (× 75) in luteal phase of menstrual cycle (17 day), showing very active glandular epithelium and much less active stroma. Incubation in glycerophosphate at pH 5, 1 hr.

cervical glands show as a rule much greater activity in the free border area, although frequently the entire cell contains a large deposit. There is slight activity in the glandular lumen itself and in the periglandular tissue. Whereas the foregoing statements apply more specifically to what obtains when glycerophosphate is the substrate, the picture with other substrates employed differs noticeably. Thus with glucose-6-phosphate an approximately reciprocal relation is encountered. With this substrate the cervical epithelium is much more inactive and the stroma in some cases considerably more active. The pattern of activity with glucose-l-phosphate as a substrate is, however, similar to that of glycerophosphate but lower. Activity with fructose-6-phosphate resembles that with glucose-6-phosphate but appears to be lower in the endocervical glands. Activity with adenosine-triphosphate was very low, in contrast to results of our studies in the alkaline range. Overnight incubation was necessary in order to obtain even slight reactions. Under these conditions some activity was noted in the cervical epithelium but not in the stroma.

Endometrium. We have had an opportunity to study acid phosphatase in the endometrium of women at different times in the menstrual cycle. Activity was greater in the glands than in the stroma; and both were higher than in other tissues (Fig. 4). Activity appears in both the nucleus and cytoplasm. In the glandular epithelium there is a slight to pronounced intensification of cytoplasmic free border activity. In contrast to our own and other studies with alkaline glycerophosphatase, there is an absence of activity in the vascular system. With glucose-6-phosphate, the one endometrium examined was again quite active; however, as in the case of the cervix, with shorter incubation time of 1-2

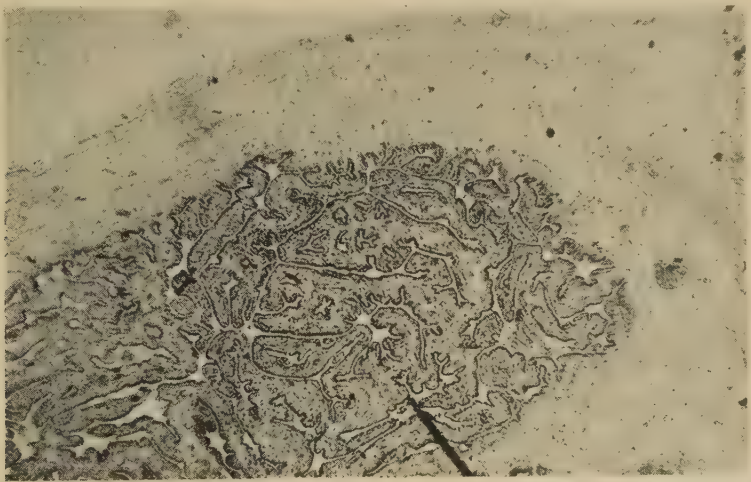

FIG. 5. Section of Fallopian tube of human (× 25) showing moderately active mucosa with activity concentrated primarily at or near free border of epithelial cells and rather inactive muscularis. Incubated as in Fig. 4.

Fig. 2-5 are from sections of fresh frozen tissues.

hours, the glandular epithelium shows a lower activity than the stroma.

Fallopian tube. The epithelial layer surrounding the lumen shows by far the greatest activity. It appears to be nuclear as well as cytoplasmic, but in the latter primarily in the free border of the cell (Fig. 5).

Discussion and summary. Biochemical and histochemical methods have demonstrated acid phosphatase activity in the vagina, exo- and endo-cervix, the endometrium, and mucosa of the fallopian tube. The enzyme is similar to that in the human prostate but the activity per mg wet weight is lower.

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Effect of Aureomycin in Rats with Chronic Alloxan Diabetes.* (20261)

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Clinical observations on several patients with severe diabetes showed that during treatment with aureomycin a marked improvement of the diabetic condition took place. Since no obvious infection was established by clinical and laboratory means in some of these patients, the impression was gained that aureomycin may have a direct favourable effect on the diabetes. These observations prompted us to study the effect of aureomycin on experimental diabetes in the rat.

Material and methods. Adult male rats weighing 130-160 g were used. Diabetes was induced by a single subcutaneous injection of 230 mg alloxan per kg body weight. Following this, half a unit of PZI was given daily subcutaneously for 7 days. Forty-four rats which were diabetic for 6 weeks were put in special metabolic cages for the following 2 weeks; the urine was collected every 48 hours, measured and examined for sugar, the daily *ad libitum* food intake was measured. The ration consisted of a mixture of ground wholewheat, ground alfalfa, bran, skimmed milk powder, salts and contained 21% proteins. Once a week the rats were weighed. Following the period of 2 weeks the diabetic rats were divided into 2 subgroups, one consisting of 25 rats (subgroup I) was given aureomycin in a dosage of 20 mg per 100 g food for 28 days, and 19 diabetic rats (subgroup II) were not given aureomycin. Nineteen normal nondiabetic male rats of the same stock and age served as control for the diabetic rats, and were given the same ration. Of these, 11 rats (subgroup III) were given the same food plus aureomycin in the same dosage as the diabetic rats, while 9 rats (subgroup IV) were not given aureomycin. In another series of experiments 8 rats which had alloxan diabetes for 6 months were given 40 mg aureomycin per 100 g food for 10 days, and 60 mg per 100 mg food for 6 days. Urinary sugar was determined daily and the bodyweight was measured once a week. *Sugar* in the urine was examined by Benedict's quantitative method.

Results. As shown in Table I the average gain in weight in the 19 diabetic rats of the first series of experiments (subgroup II) which did not receive aureomycin was 9.6 g during 28 days. During this period the average amount of sugar excreted in the urine was 3.75 g per day per animal. The average food intake in these animals amounted to 22 g per day. The average gain in weight in the 25

TABLE I. Average Gain in Body Weight, Average Daily Food Intake, Daily Diuresis and Glycosuria in Normal, Diabetic and Aureomycin-Fed Diabetic Rats.

Rats	Aureomycin	Avg gain in body wt in 28 days	Avg daily food intake	Avg daily diuresis	Avg daily sugar excretion in urine
25 diabetic	+	19.0 ± 2.9	33 ± 3.7	88 ± 13.3	5.0 ± 1.7
19 ”	—	9.6 ± 2.0	21.5 ± 2.6	73 ± 9.2	3.85 ± 1.9
11 normal	+	28 ± 1.07	15 ± 3.1	—	—
9 ”	—	28 ± 1.52	14 ± 2.7	—	—

diabetic rats (subgroup I) kept under similar conditions except for the amount of food consumed (*ad libitum*) and the addition of 20 mg aureomycin per 100 g food was 19 g per animal during the 28 days. Glycosuria in these animals increased as compared with the previous control period of the same animals and with animals of subgroup II which did not receive aureomycin. The average amount of sugar excreted per day was 5.0 g per animal as compared with 3.85 g before the administration of aureomycin and 3.75 g of subgroup II. The food intake during this period of increased glycosuria was 33 g per day per animal, as compared with 26 g during the preceding control period of the same group, and with 22 g of subgroup II during the same period of time.

The results of this experiment corroborate with those of another set of experiments with 8 chronic diabetic rats. In this experiment the diabetes was of 6 months duration. The average gain in weight during the 16 days of aureomycin administration was 18 g as compared with 1.1 g gained in 8 days preceding the administration of aureomycin. The gain in weight continued for approximately 7 days after withdrawal of aureomycin during which the average gain per animal was an additional 10 g. During the following 7 days there was a definite decrease in bodyweight averaging 11 g per animal. In this experiment also the period of aureomycin treatment was accompanied by increased glycosuria and diuresis.

The weight curve obtained in the diabetic rats with and without aureomycin were compared with that of normal non-diabetic rats (subgroups III and IV). Non-diabetic rats (subgroup III) which did not receive aureomycin showed an average increase in bodyweight of 28 g per animal during 28 days. Non-diabetic rats (subgroup IV) which received 20 mg aureomycin per 100 g food showed the same increment in bodyweight, that is, 28 g per animal during the same period of observation. The same amount of food was offered to the last two groups.

Discussion. The results of these experiments showed definitely that aureomycin has a favourable effect on the weight curve of rats with chronic alloxan diabetes. The gain in weight was substantial and statistically significant. P = <0.01. It did not reach, however, the gain in weight of non-diabetic rats during the same period of time.

Essays with various doses of aureomycin suggested that the optimum dose is 20 mg per 100 g food in a long term experiment.

This effect of aureomycin could be explained either by the "growth-promoting activity" attributed to aureomycin or to a direct and specific effect of aureomycin on the metabolism of the diabetic animal. The possibility that the "growth-promoting factor" was responsible for these gains in weight does not seem probable because the addition of aureomycin in the non-diabetic animals did not cause any added gain in body weight. The lack of the growth-promoting effect in our experiments, contrary to those reported(1-6) may be attributed to the fact that this effect was observed in very young animals only. It is however possible that the growth-promoting factor is still operative in rats of older age who are diabetic as in this experiment because of increased metabolic demands which may render them similar to young growing animals. By this assumption the growth-promoting factor would outweigh the diabetic factor resulting in a net gain in weight.

It is important to note that the increase in body weight in the diabetic rats receiving aureomycin took place in spite of increased glycosuria and diuresis. Thus there was no improvement in the diabetic state during the period of aureomycin administration as expressed by glycosuria and diuresis. The gain in bodyweight during this period in spite of increased glycosuria and diuresis can be explained by the fact that during this period the animals consumed more food. Obviously at least a part of the additional food was utilized. The cause for the increased food intake during aureomycin administration is not clear. It might be related to a possible specific metabolic effect of aureomycin.

The question of food utilization in chronic diabetic rats receiving aureomycin and the intermediary metabolic effects of aureomycin in diabetes are under investigation.

Summary. 1. Aureomycin given to chronic alloxan diabetic rats caused an average gain in body weight of 19.0 ± 2.9 g during 28 days. Control chronic alloxan diabetic rats gained 9.6 ± 2.0 g during the same period. 2. During aureomycin administration the animals consumed more food and had increased glycosuria and diuresis.

We wish to express our appreciation of the help and advice received from Professor E. Wertheimer. The aureomycin was kindly supplied by the Lederle Co.

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Effect of Oral Thyroxine and Tri-Iodo-Thyronine on Radioactive Iodine Uptake and Serum Protein Bound Iodine in Normal Human Subjects. (20262)

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Studies of synthetic sodium-levo-thyroxine have shown that, as oral medication in dosage of about 0.5 mg or less daily, it is calorigenic and clinically active(1). In similar dosage it has been shown to reduce the uptake of radioactive iodine(2). Tri-iodo-thyronine, recently identified, synthesized, and demonstrated in human serum(3), also has been shown to be clinically effective(4), and to be several times as potent as thyroxine.

This report is on the effect of these medications, given orally, on the 24-hour uptake of a 5 microcurie tracer dose of radioactive iodine* before and at the end of a week of oral medication. The serum protein bound iodine was also determined.

Procedure. The subjects were normal volunteers at their usual activities as medical students or laboratory workers. Blood was drawn for serum protein bound iodine determinations and about 5 microcuries of carrier free radioactive iodine were given; 24 hours later, the accumulation in the thyroid gland was counted and compared with a dummy. The subjects were then provided with sodium-levo-thyroxine tablets,† in dosages from 0.025 mg to 0.5 mg to take once a day by mouth for a week. On the sixth and seventh days of the week, blood was drawn for serum protein bound iodine and the 24-hour tracer study again was repeated, after a

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* Radioactive iodine received from Atomic Energy Commission, Oak Ridge.

† Generously provided by Dr. Robert P. Herwick of Baxter Laboratories, Morton Grove, Ill., by whom these studies were in part supported.

TABLE I. Effect of Oral Medication with Sodium-Levo-Thyroxine Given Once a Day for 7 Days to 17 Normal Subjects. A) before starting treatment; B) 6th day on treatment; C) 7th day after treatment discontinued; A′) uptake before treatment; B′) uptake on 7th day of treatment.

Dose, mg	Protein bound iodine, γ %			Cholesterol, mg %			I^{131} uptake, %	
	A	B	C	A	B	C	A′	B′
.025	5.2	5.0	5.0	400	318		16.7	26.0
	5.9	5.5	5.2	318	239	200	14.9	13.0
	4.9	5.7	4.2	284	326	207	30.1	28.5
	5.5	6.4		209	193		18.0	11.9
	4.8	5.7			230		21.0	29.6
.075	4.6	5.2		318	324		15.0	9.5
	3.8	4.3		253	235		27.0	9.4
.100	5.1	5.5	5.8	300	284	277	13.9	13.0
	5.5	6.0	5.3	270	290	308	31.6	22.6
	6.2	6.0	5.6	260	146	222	24.7	17.4
	5.7	5.4	4.5	230	264	276	27.0	4.0
	4.9	4.6		179	206		18.8	12.0
.200	4.6	7.1					28.0	20.7
.500	5.3	7.8					28.0	17.8
	4.9	11.0					25.0	15.3
	6.0	6.9					23.0	9.9
	4.3	6.8					26.0	12.6

background count and suitable adjustment of the tracer dose. A similar protocol was followed with tri-iodo-thyronine.‡ The tablets of this medication contained 0.035 mg. Studies were made at 6 levels of medication from 0.008 mg to 0.140 mg. It is important for future comparisons to note that the blood was drawn and the tracer given and counted within a few hours after ingestion of the medication.

Results. Depression of the uptake of the second tracer dose was found with both medications as was to be expected. The effect of the smallest doses of tri-iodo-thyronine was greater than expected. This will require further titration of the lower dose ranges of both substances to approximate the comparative effectiveness in regard to this particular action of the thyroid hormone. It seems that tri-iodo-thyronine is about ten times as active as sodium-levo-thyroxine in this respect.

With the larger dosage of thyroxine, the serum protein bound iodine rose and on the effective small dosages of tri-iodo-thyronine it fell. (Tables I and II).

Comment. The depression of thyroid gland function as measured by radioactive iodine uptake indicated in this study may be due to an action of the medication in the gland itself, as occurs in antithyroid drug therapy, or it may be due to reduction in pituitary secretion of TSH. The latter site of action conforms to the theory that thyroid function is controlled by the level of thyroid hormone in the pituitary. The present work with tri-iodo-thyronine suggests that a fall in secretion of TSH was associated with a reduction in circulating protein bound iodine. The physiological paradox of reduced circulating thyroid hormone (PBI) during administration of thyroid hormone, *i.e.*, tri-iodo-thyronine, and apparent increase of thyroid hormone action, *i.e.*, depression of uptake, is accounted for by the high biological activity of the small quantities of iodine compound involved. Whether the action of tri-iodo-thyronine is on the tissues (elevation of basal metabolic rate), on the hypothalamus, on the anterior pituitary, or on the thyroid gland itself remains to be determined.

Summary. Sodium-levo-thyroxine in dosage of 0.075 mg, orally, usually reduced radioactive iodine uptake by the thyroid of normal human subjects; this was associated with a rise of protein bound iodine when a dosage of 0.2 mg or more was given. Tri-iodo-thyronine

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TABLE II. Effect of Oral Medication with Tri-Iodo-Thyronine Given Once a Day for 7 Days to 25 Normal Subjects. A) before starting treatment; B) 6th day on treatment; C) 7th day after treatment discontinued; A′) uptake before treatment; B′) uptake on 7th day of treatment.

	Protein bound iodine, γ %			Cholesterol, mg %			I^{131} uptake, %	
Dose, mg	A	B	C	A	B	C	A′	B′
.008	4.6	3.9					18.8	2.7
	6.5	4.7					31.2	5.7
	4.8	3.5					31.6	5.9
	4.2	3.0					40.2	15.9
	3.6	2.9					18.8	6.2
.0175	4.7	4.3	5.7	276	276	295	22.0	13.3
	5.4	4.4	4.4	165	146	177	26.9	15.7
	5.0	4.0		259	227		21.0	2.3
	5.1	4.4	3.9	192	162	200	14.5	7.0
	5.0	4.1	3.8	565	543	520	21.3	4.6
	4.0	4.0	6.5	317	295	350	16.0	4.3
.035	4.2	4.2	6.4	234	212	235	17.0	2.7
	4.6	3.4	4.9	213	162	259	23.0	5.0
	5.4	2.7		260	343		20.0	10.0
.070	5.7	5.2		256	262		28.0	3.1
	3.8	5.0	3.8	244	192		20.0	5.0
	5.0	3.0	4.2	200	204	238	18.0	4.0
.105	4.0	4.9		312	294		13.0	6.7
	4.0	3.9	3.5	272	192	273	32.0	8.9
	4.4	2.9	4.3	192	227	224	13.0	2.0
.140	5.8	4.7		235	194		18.9	6.5
	6.4	4.9	5.1	214	322	228	19.4	6.5
	5.3	5.7	4.6	255	208	277	30.0	5.4
	4.6	5.1		200	208		14.0	8.6
	3.2			255			19.0	4.3

in dosage as low as 0.008 mg orally also reduces the uptake. This is associated with a decrease in serum protein bound iodine.

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Effect of Piromen on Survival of Irradiated Mice.* (20263)

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Piromen‡ stimulates a leukocytic response very similar to that produced by treatment with ACTH(1-3). The mechanism of Piromen stimulation is unknown, but it is believed that the drug affects directly or indirectly the adrenal cortex(4). There is evidence, how-

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‡ Trade name for a sterile non-protein, non-anaphylactogenic bacterial pyrogen. Furnished through the courtesy of Dr. William F. Windle, Baxter Laboratories, Morton Groves, Ill.

ever, that its action is not identical with that of ACTH(5-7). It has also been shown to induce changes in the connective tissue mast cells(8). Jacobson, *et al.*(9) have postulated that it may be possible to stimulate the production of the factor responsible for rapid recovery from radiation injury. This factor seems to be intimately associated with the spleen. Since Piromen stimulates hemopoiesis and produces hyperplastic myeloid spleens, it seemed probable that this substance might effect an increase in production of the radiation-recovery factor. This possibility, plus the demonstrated importance of the role of adrenal response in alleviation of radiation sickness(10), prompted the inquiry into the possible protective action of Piromen.

Materials and methods. A total body dose (skin) of 550 r was delivered in each experiment by a deep therapy General Electric Maximar X-ray machine. Factors used were: 220 kvp, 15 ma, 1.0 mm Al, 0.5 mm Cu and 0.5 mm Cu equivalent (inherent in machine) filters, 50 cm target-center of animal distance, 1.25 mm Cu half-value layer, and 49.2 r per minute dose rate. An Oddman localizer was used to determine the exact location of the 15 cm square field. To avoid possible stress and complications due to hormonal influences, the animals were neither anaesthetized nor closely confined during irradiation. With small animals such as mice, body irradiation may be considered to be uniform throughout. The irradiation chamber was constructed of ⅛ inch lucite with ample ventilation ports. The total dose was determined by summing repeated r-readings with a 100 r Victoreen Condenser meter and correcting for temperature and barometric pressure. Animals were maintained in an air-conditioned room at 25°C, and allowed free access to Purina Laboratory Chow and water (controls), or Piromen-treated water. Preliminary experiments with C3H and several Swiss Albino strains indicated that results were comparable. The HN strain of Rockefeller Institute Swiss albino mice was used in all experiments reported here. *Exp. I.* Thirty-two 10-week-old mice (18 males, 14 females) received an average daily dose of 0.37 γ of Piromen, calculated on the basis of the amount added to drinking water, 4 successive days prior to irradiation (total dose, 1.48 γ). The control group, which received no Piromen, consisted of 32 animals (18 males, 14 females) of the same age. *Exp. II.* Another experiment was conducted in which Piromen was administered intraperitoneally. Seven-week-old mice were used. Group 1 (37 mice, 25 males) received 0.1 γ Piromen/day for 5 days before irradiation. Group 2 (37 mice, 25 males) received the same dose of Piromen for 5 days after irradiation, and group 3 (39 mice, 26 males) received one ml physiological saline 5 days before and 5 days after irradiation. Group 4 (10 mice, 5 males) received 0.1 γ Piromen/day for 10 days, but was not irradiated. *Exp. III.* The effect of post-irradiation treatment with larger Piromen doses was determined by using 2 groups of 8-week-old mice. Twenty-six mice, half male and half female, were employed in each group. Due to the fact that there is a decrease in water consumption immediately after irradiation, Piromen was administered as follows: Group 1 received 0.5 γ intraperitoneally immediately after exposure and thereafter the daily dose, calculated on the basis of Piromen contained in water consumed, was 0.34 γ/day/mouse for 6 days (total dose 2.55 γ). Mice of group 2 were given an injection of saline containing no Piromen.

Results and discussion. Results of these experiments are presented in Table I.

When Piromen was administered intraperitoneally before irradiation, as in Exp. II, it had a significant protective effect. Piromen, even at twice the experimental dose did not impair health or survival. Post-irradiation treatment, on the other hand, significantly decreased survival time. When the post-irradiation dose was increased, as in Exp. III, all Piromen-treated animals died within 11 days, whereas 4 of the control group survived at least 30 days. This is a significant difference and indicates how a small change in dosage may affect survival. No sex differences were observed in any experiments.

It appears that as regards survival the primary action of Piromen is very similar to that of ACTH. As in the case of ACTH treatment under similar conditions(11), a protective ac-

TABLE I. Survival of X-Irradiated Piromen-Treated Mice.

Exp. No.	Treatment	Survival Fraction*	%	Mean	Significance†
I	Small oral Piromen doses before irradiation (.37 γ/day for 4 days)	13/32	59.4	14.15	P >.05
	Control (no Piromen)	13/32	59.4	12.31	—
II	Piromen inj. i.p. before irradiation (0.1 γ/day for 5 days)	22/37	40.6	19.00	P <.05
	Piromen inj. i.p. after irradiation (0.1 γ/day for 5 days)	32/37	13.5	12.95	P <.05
	Control, saline only, inj. i.p. 5 days before and 5 days after irradiation	30/39	23.1	14.54	
	Piromen control, (no irradiation) (0.1 γ/day for 10 days)	10/10	100	30.00+	
III	Larger Piromen dose after irradiation (1 ml saline containing 0.5 γ/ml i.p. 1st day after irradiation, then 0.34 γ orally for 6 days)	26/26	0	8.04	P <.05
	Control. One ml saline i.p. first day after irradiation. No Piromen	21/26	19.0	16.65	

* No. died/total.

† No. surviving as compared with control in each experiment, based on "t" test.

tion is obtained, but post-irradiation treatment lowers the number of survivors. Perhaps Piromen and ACTH exert an additive deleterious effect upon tissues already damaged by irradiation. The leukocytosis-promoting factor, isolated by Menkin(12), which produces a pharmacological effect somewhat like that of Piromen, has been shown, however, to have no effect upon survival of X-irradiated mice(13).

Summary. Intraperitoneal injection of 0.1 γ Piromen/day/mouse for a 5-day period effected a significant increase in the mean survival time of X-irradiated (550 r) mice of the HN Rockefeller Institute strain. Piromen administered with the drinking water at the rate of 0.37 γ/day/mouse for 4 days failed to significantly alter survival time. This may have been due to the small dose actually absorbed. Administration of a larger dose (total dosage, 2.55 γ) after irradiation resulted in fewer survivals than in the untreated controls. The action of Piromen following irradiation appears to be similar to ACTH with respect to survival. The dosage of Piromen is apparently of critical importance.

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Arginase Activity in Vitamin E-Deficient Rats.* (20264)

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It is well known that vit. E is necessary for normal gonadal function in certain species, and that in the rat, deprivation of the vitamin produces structural changes in the testes, with characteristic degeneration in the germinal epithelium(1). A similar need for arginine is not as well established, but is indicated by the observation that deficiency of this amino acid results in testicular degeneration resembling that of vit. E-deficiency(2). Moreover, in a patient with myotonia atrophica, associated with testicular atrophy, an abnormally low urinary excretion of 17-ketosteroids was increased following ingestion of arginine(3). Whether the functions of vit. E and arginine are related to each other is not known, but such relationship is at least suggested by the fact that the deleterious effects of vit. E-deficiency on the testes are not seen in the mouse which is able to synthesize arginine more readily than the rat(4). Therefore, it appeared of interest to determine whether the structural changes in the testes of vit. E-deficient rats could be delayed or prevented by the administration of arginine. In addition, since the activity of certain enzymes, for example, transaminase(5) may be altered in vit. E-deprivation, it seemed further of interest to ascertain whether arginase activity also is affected in the tissues of the deficient rat. Definitive data have not heretofore been reported. Williams and Watson(6) studied the arginine content of the tissues and the arginase activity of the liver of partially vit. E-deficient rats, but their results permit no conclusions regarding the possible effects of complete or nearly complete deprivation of the vitamin. More recently, Cruz-Coke and his coworkers(7) found tocopherylphosphate to inhibit the arginase of liver homogenates and of partially purified arginase preparations, but as has been the case with many experiments in which tocopherylphosphate was employed *in vitro* the changes observed probably were due to the phosphate grouping rather than to the tocopherol itself. In another series of investigations(8,9), the same workers observed that tocopherylphosphate given orally to the frog or rat produced no change in the arginase activity of the liver; similarly, administration of arginine was without effect in the rat. The animals employed by these investigators were not maintained on vit. E-deficient diets, and in consequence, the observations are of doubtful pertinence to the problem that served as the basis of the investigations reported on here.

Methods and material. Thirty male rats were segregated into 3 groups at 21 days of age and maintained on diet No. 30 of Mason and Bryan(10). One group was given daily supplements of synthetic α-tocopherol calculated to be sufficient for normal development. The other 2 groups were deprived completely of tocopherol but the diet in one of these groups was supplemented with L (+) arginine monohydrochloride. The dosage of arginine was increased gradually over a period of 6 weeks from an initial level of 100 mg daily to 600 mg per day, the latter supplementation being continued until the animals were sacrificed. The growth of the animals was followed by weekly weighings while the development of testicular changes was ascertained by periodic biopsies(1,10). The histologic examinations were started after the animals had been on the experimental diet for about one month and were continued until the experiments were terminated. At monthly intervals, one animal from each of the 3 groups was selected for biopsy, until all of the animals had been subjected to this study. The composite data gave a representative record of the development of the testicular changes occurring in each group. Liver and kidney arginase was determined by the

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method of Kochakian(11). In this procedure, which employs a veronal-buffered arginine substrate, the urea liberated by the action of the arginase is treated with urease and the resulting ammonia is collected in a boric acid solution. Titration is performed with 0.015 N HCl and estimation of arginase is derived from a calibration curve constructed according to the directions of Edlbacker and Röthler (12). Homogenates of liver and kidney were diluted and aliquots representing 0.38 mg and 15 mg respectively, of wet tissue, were incubated. All incubations were performed in duplicate.

Results and discussion. The growth curves of the animals in all 3 groups were similar. Gross appearance of muscular dystrophy was observed in only a few animals in which weakness of the hind legs developed after deprivation of tocopherol for approximately one year.

Photomicrographs of the testes are not presented here but histologic analysis indicated a normal picture for the control rats, whereas in all of the animals maintained on the vit. E-deficient diet, testicular changes were apparent. In about 70% of these animals, the testicular degeneration was severe and it was evident that the administration of relatively large doses of arginine had afforded no protection against these histologic alterations. As a matter of fact, histologic examination of the seminiferous tubules of the rats that were given arginine suggested a more rapid loss of active sperm and an earlier production of giant cells than in the animals that had been given no supplements of arginine. Sections of skeletal muscle, liver and kidney of the animals in all 3 groups were normal.

Data on the arginase content of liver and kidney tissue for the 3 groups of animals and the wet weight of these organs are given in Table I. It can be seen that liver homogenates from normal, vit. E-deficient and arginine treated E-deficient rats split approximately identical quantities of arginine after one hour of incubation. In conformity with the now commonly used procedure, these results have been expressed in terms of arginase units. Since the total weights of the organ were essentially the same in the animals of all 3 groups, the total arginase activity also was essentially alike.

TABLE I. Average Arginase Units per Gram Tissue.*

Diet	No. animals	Avg total wt, g	Avg arginase per g, units
	Liver		
Normal	7	10.7±1.8†	1264±415.9
Vit. E-deficient + arginine	9	11.1±2.0	1143±189.31
Vit. E-deficient	11	10.9±1.7	1172±290.1
	Kidney		
Normal	7	1.1±0.2	14.6±5.8
Vit. E-deficient + arginine	9	1.4±0.2	15.3±3.0
Vit. E-deficient	11	1.4±0.2	12.5±3.1

* An arginase unit = ml 0.015 N HCl needed to neutralize NH_3 released from urea derived from 1% arginine substrate during one hr incubation at 38°C with 0.38 mg liver or 15 mg kidney (wet wt).

† Stand. dev. from the mean.

Kidney homogenate incubations revealed a similar situation as shown in Table I. Whereas the control animals and those deprived of tocopherol but fed arginine may appear to have decomposed arginine to a slightly greater extent than that exhibited by kidney preparations from E-deficient rats not administered arginine, statistical analysis indicated that such difference was not significant.

Summary. 1. Male rats were raised on a vit. E-deficient diet; some animals were given daily supplements of arginine monohydrochloride. 2. Arginase activity of liver and kidney determined by the method of Kochakian. 3. There was no difference in arginase content of livers and kidneys of animals maintained on a vit. E-supplemented diet, a vit. E-deficient diet and vit. E-deficient diet containing a supplement of arginine. 4. Administration of arginine did not delay or modify the development of testicular degeneration resulting from vit. E-deficiency.

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Influence of the Drug DDD on Adrenal Cortical Function in Adult Rats.*†
(20265)

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A recent report by Nichols and Gardner(1) described the effect of the drug DDD (2, 2-bis (parachlorophenyl) 1, 1-dichloroethane) on the adrenal cortex of the dog. These investigators found that the oral administration of the drug for periods up to 8 weeks resulted in a sensitivity to insulin indicating that the adrenal cortex might be involved. This supposition was confirmed by the marked atrophy of the adrenal cortex found on histological examination. In a later report(2) Nichols and Sheehan reported that DDD would alleviate the diabetes produced by alloxan, again implicating the adrenal cortex. However, earlier workers in the field do not agree. Haag *et al.*(3) reported no adrenal damage when DDD was fed at a level of 1200 PPM to rats and Lillie *et al.*(4) found that rabbits did not suffer adrenal atrophy when fed at a level of 0.1% DDD in the diet. In view of the confusion in the literature and the relatively small amount of data available it was decided to test the effect of DDD in rats, determining the effect of the drug on eosinophil count, the level of Na and K in the blood and urine, the insulin sensitivity, and the response to stress.

Materials and methods. Male white rats of the Sprague-Dawley strain averaging about 100 g in weight were used in all experiments. Each animal was tested before use by the blood sugar response to administration of 0.1 unit of insulin per 100 g body weight. The fasting blood sugar was also determined and all animals with abnormally high fasting blood sugar levels or with abnormal insulin sensitivity were eliminated from the experiment. Every animal was also tested for eosinophil response to ACTH before testing for insulin sensitivity. Each animal was given 1 unit of ACTH intraperitoneally and eosinophil counts were made 4 hours following the injection. All rats which did not show a drop in eosinophil count of at least 80% were arbitrarily discarded. The animals were then divided into a normal group and a group receiving 0.1% DDD in the diet. The amount of diet consumed by each animal was determined. Both normal and experimental groups consumed the same amount of diet. At intervals of 1 week, the controls and the group fed DDD were tested for insulin sensitivity by the intraperitoneal administration of 0.1 unit insulin/100 g body weight. The blood sugar was determined on tail vein samples by the Somogyi method at ½, 1, 2, 3 and 4 hours after injection. When the animals developed insulin sensitivity, they were placed in metabolism cages and urine collections were made. After a 3-day collection the animals were starved for 3 days and collections were

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† Part of this data presented at April, 1953, meetings of Federation in Chicago.

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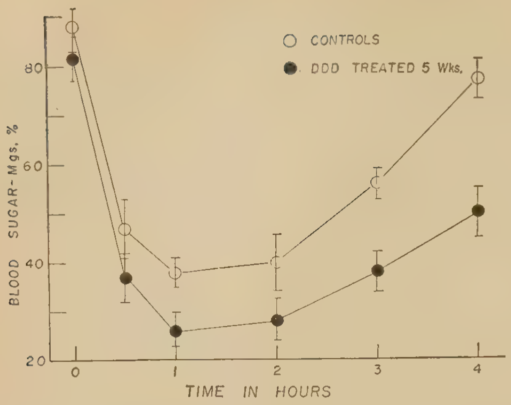

FIG. 1. Influence of DDD on insulin sensitivity curves of adult rats. All animals given 0.1 unit regular insulin/100 g B.W. Bars represent ±1 S.D. 8 animals used in each group.

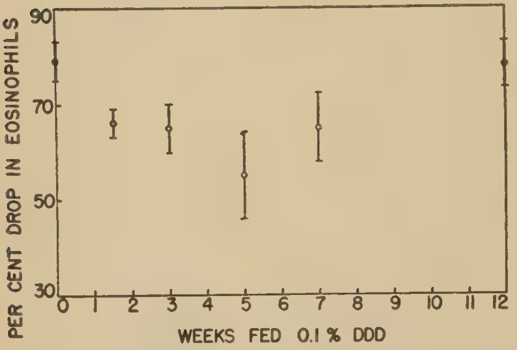

FIG. 2. Relationship between length of time that rats received a DDD diet and decrease in circulating eosinophils when rats were given 1 unit of ACTH intraperitoneally. All counts made 4 hr after inj. Barred lines indicate ±1 S.D.

again made on the last 2 of the 3 days. The animals were then fed for 3 days and on the fourth day an eosinophil count was made as described above. The phloxine method was used with all due precautions(5). After the count, the animals were etherized, bled by heart puncture and autopsied. Organ weights were determined, histological specimens were taken and the urine and plasma were analyzed for Na and K with the Perkin-Elmer flame photometer. Animals selected for the stress experiments were placed on a diet containing 0.1% DDD for a period of 5 to 6 weeks. At the end of this time the controls and the animals fed DDD were shaved, placed in separate cages in a cold room at 3°C and the survival time of each group was determined. Other groups of controls and DDD treated rats were given 1 unit of ACTH per day for 2 days prior to submission to the cold test.

Results and discussions. The data presented in Fig. 1 demonstrate that the animals treated for 5 weeks with 0.1% DDD in the diet develop moderate insulin sensitivity. Animals treated for shorter lengths of time do not develop as marked a sensitivity. The barred lines indicate the range of variation in blood sugar levels and demonstrate significant differences ($P < 0.05$) between the controls and the animals treated with DDD. It is interesting to note that several of the DDD treated group developed convulsions and that one animal died when given a dose of insulin which produced no visible signs in the control group. This insulin sensitivity is characteristic of animals and humans with impaired adrenal cortical function. This indication that the compound might be affecting the "sugar" hormones of the adrenal cortex (*e.g.* cortisone-like substances) was further tested by injecting both the control and DDD treated animals with small doses of ACTH and measuring the per cent fall in eosinophils after a 4-hour interval. The data presented in Fig. 2 reveal that the control animals exhibited the characteristic response to ACTH in that the eosinophils fell an average of about 82% in the 4-hour interval. The eosinophil drop in the animals treated with DDD for 5 weeks was about 54% in the same time interval and with the same dose of ACTH. The difference is significant ($P < 0.05$). This observation indicates that DDD does prevent the elaboration of cortisone-like substances to some degree but that the inhibition is not complete.

Fig. 2 also shows the variation in eosinophil response with time on the diet. The eosinophil response becomes minimum at the same

TABLE I. Effect of Feeding DDD for 5 Weeks on Survival Times of Adult Rats Exposed to 3°C until Death.

Treatment	No. of animals	Survival time in hr
Control	12	10.3 ± .7*
DDD 5 wk	13	6.1 ± .8
" + ACTH	10	5.6 ± 1.2
Normal + ACTH	8	12.3 ± 1.4

* Stand. dev.

TABLE II. Influence of 0.1% DDD on Na and K Excretion, Uric Acid/Creatinine Ratio and Adrenal Weight of the Adult Rat.

Wk on DDD	No. of animals	Uric acid/creatinine ratio	Urine			Plasma		Adrenal wt, mg/100 g body wt
			Vol Ml/day	Na Meq/liter/day	K Meq/liter/day	Na Meq/liter	K Meq/liter	
Control	18	.78 ± .10*	16	22.4 ± 2.1*	38.5 ± 4.7*	144 ± 2	5.0 ± 1	15.4 ± 1.1*
1½	8	.78 ± .08	17	24 ± 4.6	36.4 ± 5.3	146 ± 1	6.0 ± 1	21.3 ± 1.2
3	8	.48 ± .08	15	28.6 ± 3.7	52.8 ± 8.6	144 ± 3	5.0 ± 1	20.1 ± 1.4
5	12	.45 ± .09	18	19.9 ± 2.6	40.6 ± 7.1	144 ± 2	5.0 ± 1	19.2 ± 1.7
12	8	.56 ± .12	18	17.5 ± 5.2	44.8 ± 7.3	145 ± 2	5.0 ± 1	16.7 ± 1.2

* Stand. dev.

time that the insulin sensitivity is maximum. This would be anticipated on the assumption that DDD is interfering with the production and/or release of certain adrenal steroids. The decrease in eosinophil response declines to a minimum at the fifth week and thereafter the response returns toward normal. The barred lines in the figure indicate the range of the data. It becomes obvious that the difference between the controls and the animals fed DDD for 5 weeks is significant ($P < 0.05$) while the comparison between the controls and the animals fed DDD for longer or shorter periods of time is not so significant.

The data in Table I indicate that those animals which have received DDD for 5 weeks have a greatly decreased response to stress as measured by the cold test. Normal control animals can survive extreme cold for a period of about 10 hours. The animals treated with DDD on the other hand can survive the same degree of cold for only 6.5 hours. This difference is statistically significant ($P < 0.05$). Any single test would not be considered of great importance, but when animals fed DDD for 5 weeks have a decreased response to cold, a decreased eosinophil response and an increased insulin sensitivity and when all of these responses are assumed to be under the control of the "gluco-steroids" the evidence becomes more conclusive. The adrenal gland is no longer able to produce the material which enables the organism to respond normally to the above tests. The site of destruction or lack of production must be at the adrenal level because the administration of ACTH does not restore the animal to normal in the stress test (Table I).

Further evidence of the impairment of adrenal cortical function was found in the uric acid/creatinine ratio (Table II). Selye (6) has stated that an increase in the production of steroids (*e.g.* under stress) will produce a rise in uric acid excretion and an increased ratio. Conversely, a decrease in the normal level of steroid production should produce a decrease in this ratio. Such a decrease is found in animals treated for 5 weeks with DDD.

In order to test the ability of the animals treated with DDD to conserve Na and excrete K, the rats fed DDD for 5 weeks were starved during urine collection periods. The data in Table II demonstrate that both groups were essentially equal in their ability to excrete K and conserve Na. The plasma Na and K were within normal limits in both the control and DDD treated groups.

Nichols and Gardner observed that the adrenals of dogs fed DDD atrophy in the inner zones of the cortex with little or no change in the zona glomerulosa. The data obtained in the rat in the present experiments do not confirm this observation. The data in Table II on the weight of the adrenal glands in rats fed DDD show clearly that the adrenal increases in size from the normal to a maximum size after 1½ weeks on the DDD diet, after which time the adrenals decrease in size. This is in direct contradiction to the data obtained in the dog but confirms the report of Haag *et al.*(3) that DDD fed to rats produced no adrenal change. Haag did not investigate the physiological changes in his animals and determined the adrenal weight only after the animals had been on the diet for several weeks. Histological sections of the adrenals in our experiments show no significant change in cell structure, despite the alteration in size and function and they indicate that there is no change in the size of the glomerulosa.

Summary. Adult rats fed the drug DDD rapidly develop signs of some adrenal dysfunction; decreased eosinophil response, decreased uric acid/creatinine ratio, increased insulin sensitivity and decreased response to stress of cold. The urinary and plasma Na and K are not affected.

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Localization of Glycolytic and Respiratory Enzyme Systems on Isolated Mouse Brain Mitochondria.* (20266)

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The Krebs cycle has been demonstrated using cyclophorase preparations, *i.e.*, suspensions of washed homogenate pellets(1). On the other hand, the investigators who have worked with isolated mitochondria prior to this time have demonstrated many of the Krebs cycle reactions, but never the malonate-sensitive oxidation of pyruvate denoting the complete cycle(2-9). These findings are in accordance with the fact that isolated mitochondria have not previously been reported to show the reversible staining with Janus green B which is characteristic of mitochondria *in vivo*(10). The absence of this special staining reaction indicates that the functional intactness of these mitochondria was apparently of too low a degree for them to carry the complete Krebs cycle. Glucolysis has been studied in extracts of various tissues(11-15). This process has, however, never before been demonstrated for isolated cell constituents used singly or in combination. Glycolysis, with hexose-diphosphate in the presence or absence of glucose as substrate, has been reported to occur in the supernatant(16-18); supernatant plus submicroscopic particles(6); or in the supernatant, in the submicroscopic particles, and in the mitochondria(19). No report on the Janus green B staining reactions of these preparations was given. The present paper briefly presents data which demonstrate that both these enzymic sequences occur in balanced quantities on mouse brain mitochondria isolated and washed in 0.25 M sucrose.

Materials and methods. Webster strain mice were killed by cervical dislocation and the entire brain immediately removed. The tissue was collected in ice-cooled 0.25 M sucrose solution at pH 7.4-7.7. Razor-cut slices were made from brains in a small amount of fresh sucrose medium in a petri dish set in ice. Slices were drained of solution, weighed and placed directly in manometric vessels. Brain tissue used to prepare mitochondria and supernatant was homogenized in a pyrex glass test tube fitted with a plastic plunger, and suspended in 9 volumes of fresh sucrose medium. It was then subjected to one 45-60 second clearance run at up to 7000 g, in a refrigerated centrifuge, to remove nuclei, whole cells and cell fragments.

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* These data are from a thesis to be presented to the Faculty of the Graduate School of Georgetown University by M. L. Hesselbach in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The volume of the remaining suspension was measured and it was then centrifuged at 25,000 g for 20 minutes to yield a first supernatant and a mitochondrial pellet. The mitochondria were washed by resuspension to their original volume with sucrose medium and centrifugation for 60 minutes at 25,000 g. The particulates were ready for use when this pellet had again been resuspended to its original volume in sucrose solution. The first supernatant was also centrifuged for 60 minutes at 25,000 g to clear it of the remaining tissue particles. The relative purity of the mitochondrial preparations[‡] which carried out the described activities must be emphasized. The fractions were checked microscopically. Most of the cellular debris was carefully removed in the clearance run. The use of a non-ionic medium throughout the preparative steps minimized the possible aggregation of submicroscopic particles to a point where they would sediment in the mitochondrial pellet. It also minimized the direct association of these particles with the mitochondria. Furthermore, the mitochondria were washed in order to remove adherent material. The large mitochondrial pellet was cream-colored and opaque as is characteristic of these particulates. The long, high speed centrifugation given the supernatant to remove tissue contaminants, yielded, in contrast to the above, a small, semi-transparent, cream-colored gelatinous pellet, typical of the submicroscopic particles present in these preparations. Tissue was ice-cooled throughout all manipulations until the Warburg vessels were placed on manometers. Gassing was done with manometers in place on a 38°C bath. About 20 minutes elapsed between the time the vessels were taken out of the ice-bath until readings were begun. Gas exchange under aerobic (100% O_2) and anaerobic (95% N_2/5% CO_2) conditions was measured by the usual manometric technic. The coenzyme and other chemicals used in these experiments were obtained commercially. Nitrogen content was measured by the Kjeldahl technic.[§] The lactic acid which accumulated both anaerobically and aerobically was measured chemically by the Barker-Summerson method[§](20). (Anaerobic lactic acid formation was also measured manometrically, as these vessels contained bicarbonate.)

Results and discussion. Glucose was used as substrate in all experiments reported here. The results are summarized in Table I. It can be seen that only the preparations which contained mitochondria showed oxygen consumption in the presence of glucose. That these particulates, even when isolated and washed, carried the complete glycolytic system was shown by the accumulation of lactic acid under both aerobic and anaerobic conditions. That they carried, in addition, the complete Krebs cycle was demonstrated by their sustained activity under aerobic conditions, and by other experiments which proved that oxygen consumption under these conditions was malonate-sensitive.

In contrast, the supernatant did not show any oxygen consumption with glucose as substrate. It did, however, produce lactic acid aerobically and anaerobically.

Results of the lactic acid determinations are presented both on a whole-tissue-equivalent basis (total lactic acid formed per vessel) and on a nitrogen basis; so that the two sets of relationships may be observed.

Mention should be made of the submicroscopic particle pellet described in the materials and methods section. When it was tested for glycolytic and respiratory activity with the same cofactors as supplied to the other fractions, neither enzyme system could be demonstrated. Similar fractions have been found by other workers to carry various single enzymes and component chains from both systems. Since their enzyme content varies with method of preparation, it is improbable that they occur preformed in the intact cell.

It was noteworthy that a quantitative rela-

[‡] Proof of the good condition of the isolated mitochondria was that they stained and destained with 0.01% Janus green B. (Unpublished work of Showacre and duBuy, this laboratory.)

[§] The authors are greatly indebted to Mrs. Evelyn Peake, who performed the nitrogen analyses, and to Mr. Chas. Remsburg, who carried out the lactic acid determinations, both of the Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

TABLE I. Aerobic and Anaerobic Glucose Utilization by Mouse Brain Preparations.

		Gas phase							
		100% O_2				95% N_2/5% CO_2			
Fraction, duration	Substrate	µl O_2 /mg N	γ lactic /vessel	γ lactic /mg N	Final pH	µl CO_2 /mg N	γ lactic /vessel	γ lactic /mg N	Final pH
Slices, 120 min.	0	129, 96	38*	41*	7.24	45, 40	124	135	7.82
	Glucose	223, 230	1548	1681	6.53	208, 201	1464	1593	7.26
Mitochondria, 120 min.	0	78, 84	70*	103*	7.41	65, 59	63*	92*	7.90
	Glucose	274, 273	332	487	7.20	198, 200	742	1090	7.59
Supernatant, 120 min.	0	10, 9	58	145	7.22	99, 95	89, 85	223, 213	7.90
	Glucose	7, 5	1616	4050	6.78	326, 352	1260, 1140	3158, 2860	7.51
Mitochondria and supernatant, 90 min.	0	106, 91	71*	56*	7.24	11, 12	129	101	7.79
	Glucose	179, 226	1320	1031	6.53	129, 122	1232	965	7.21

* These preparations did not show the characteristic purple in the lactic acid test, but only the slight green interference color given by fructose.

The main compartment of each vessel contained: 8.7 mg K_2HPO_4, 0.43 mg diphosphopyridine nucleotide, 5.1 mg niacinamide, 0.85 mg adenosine triphosphate and 0.825 mg $MgSO_4$. The anaerobic vessels contained, in addition, 4.0 mg $KHCO_3$. The center well of each aerobic vessel contained 0.1 ml 2N KOH. 40 mg wet wt of slices in the indicated vessels. Concentration of fractions in each vessel was comparable to the calculated concentration of these fractions in the vessels containing 40 mg of slices. 6.7 mg anhydrous glucose was added last, directly to main compartment of indicated vessels. Tissue and additions were prepared in 8.6% sucrose to give a final volume of 1 ml per vessel. Gas values are based on individual vessels. Lactic acid and pH determined on the contents of a number of the same vessels.

tionship existed between glycolysis and respiration on the mitochondria. Under aerobic conditions, only the particulates prevented the accumulation of lactic acid to the extent that they respired (2.5 µ M O_2 equivalent to 1 µ M pyruvate). This relatively good balance between glycolysis and respiration allowed mitochondrial metabolism of glucose to continue for as much as 6 hours, in the presence of air or oxygen. The other fractions in contrast, formed as much lactic acid aerobically as anaerobically. (See columns 5 and 10 of Table I for pH values.)

It is reasonable to assume that some of the Krebs cycle components had washed off, as diphosphopyridine nucleotide and adenosine diphosphate had to be supplied for maximum activity. However, the enzymes of the cycle must have remained bound to the mitochondria to a great degree, since neither glucose nor pyruvate, sparked with traces of succinate, nor succinate itself, in substrate amounts, was respired by the supernatant. This conclusion is further supported by the fact that the respiratory activity of the isolated mitochondria approached that of the slices. The activity of the Krebs cycle was here dependent on an adequate supply of substrate from the glycolytic system. Since the high percentage of Krebs cycle activity retained on the isolated mitochondria was maintained by their glycolytic system, it follows that a major part of this system was also bound on the washed particulates.

The balance between the glycolytic and Krebs cycle activities may very well be dependent on the orientation of the components of these systems on the mitochondrial structure. It is recognized that some changes occur in the mitochondria during isolation. These changes might result in sufficient disorientation of glycolysis to show the rather small excess of glycolytic activity over Krebs cycle activity of the washed mitochondria. The glycolysis carried out by the supernatant may then represent the activity of only a small fraction of the total system originally on the mitochondria, now no longer limited by the coordinating effect of the particulate structure. The particulate-bound glycolytic and respiratory systems might function like two endless belt systems, linked by pyruvate.

Summary. 1. Washed mouse brain mitochondria carried out the complete enzymic conversion of exogenous glucose to carbon dioxide and water. Glucolysis took place anaerobically and aerobically, with the production of lactic acid. Under aerobic conditions glucose was oxidized by means of a malonate-sensitive cycle. The accumulation of lactic

acid was correspondingly decreased. 2. In contrast, the supernatant carried out anaerobic and aerobic glucolysis, but not respiration of glucose. The submicroscopic particle fraction did not carry out the reactions of either system.

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Nature of Growth Inhibitor in Some Mammalian Sera For Pleuropneumonia-Like Organisms of Human Origin. (20267)

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The inhibitory effect of some mammalian sera, when used in a concentration greater than 10%, on the growth of pleuropneumonia-like organisms (PPLO) has been noted by several investigators. Rabbit, sheep, and horse sera are satisfactory supplements but guinea pig, steer, and cow sera are inferior for the cultivation of the bovine pleuropneumonia organism(1). Growth of this organism was partially inhibited by 20% and completely inhibited by 50% horse serum. Edward(2), likewise, found that 17 strains of pleuropneumonia organisms of various origins grew poorly when bovine serum was used as a supplement. Morton, Smith and Leberman (3) compared various mammalian sera as supplements for the cultivation of human strains of PPLO. Bovine serum and citrated human plasma added to the basal medium in concentrations exceeding 10% were definitely inhibitory. Separation of the active growth factor in bovine serum eliminated this growth inhibitor(4). Since the fractionation of serum to obtain the growth factor removed the globulins, it was speculated that the inhibitor might be associated with antibody against PPLO. This report shows that the inhibitory component of bovine plasma is associated with alpha-globulin and the inhibitory action is enhanced by the presence of complement.

Materials and methods. The method employed for measuring the inhibitory activity of various fractions was essentially the same as previously reported(4). The preparations to be tested were incorporated into Bacto-PPLO agar with added 1% fraction A derived from bovine serum. Fraction A is the serum fraction containing the protein growth factor required by some strains of PPLO of human origin and has been described previously(4).

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Duplicate plates for each preparation were inoculated with 0.05 ml of the supernates after grinding agar blocks containing the PPLO colonies in Bacto-heart infusion broth. The inoculum for each strain in any one experiment was derived from the same supernate. The inocula were spread evenly over the plates with a bent glass rod. Two-day-old stock cultures of 4 representative human strains, 07, 09, 48 and 60, were used for preparing the inocula. The stock cultures were maintained on Bacto-PPLO agar containing 1% fraction A. Incubation was carried out aerobically for 72 hours at 37°C. The diameters of ten randomly selected colonies on each plate were measured by means of an ocular micrometer under the low power of the microscope. The *amounts* of the various bovine serum or plasma fractions added to the test medium were such as to give a final concentration equivalent to the amount of the test fraction found in 30% whole serum. A 30% final concentration was selected because with this amount definite inhibition of growth was easily detectable. Bacto-PPLO agar plates containing 30% final concentration of whole bovine serum and containing 1% fraction A were set up as controls. The agar concentration in all cases was kept at 1.2% so as to eliminate the effect of agar concentration on the size of the developing colonies. *Four fractions* of bovine serum were prepared by the ammonium sulfate precipitation method of Cohn *et al.*(5). These fractions consisted of the proteins separating between the following percentage saturation with ammonium sulfate (Merck reagent): 0-34%; 34-40%; 40-50%; and 50-60%. The precipitates were washed in the corresponding solutions of ammonium sulfate, redissolved in distilled water and dialyzed against frequent changes of distilled water for 72 hours in the refrigerator. Whole bovine serum was dialyzed in the same manner. Sterilization of all fractions was accomplished by filtration through Selas 02 unglazed porcelain filters. The *role of complement* in the inhibition of the growth of PPLO by whole serum and its globulin components was studied. Whole bovine serum inactivated by heating at 56°C for 30 minutes, inactivated bovine serum plus guinea pig complement (Lyovac), alpha globulin (bovine plasma fraction IV, Armour) plus complement, beta globulin (bovine plasma fraction III-1, Armour) plus complement, and gamma globulin (bovine plasma fraction II) plus complement were added to portions of the basal medium. The final concentration in the medium of alpha-globulin was 0.28 g %, of beta-globulin 0.26 g %, and of gamma-globulin 0.22 g %. Complement was added to give a 1:30 final dilution. The complement was the last component added to the medium. The plates were poured immediately and were inoculated as soon as possible in order to minimize inactivation. One per cent fraction A was added to all of the test media except those containing whole serum since the various globulin fractions will not support growth of PPLO. Normal bovine serum, complement plus fraction A, and fraction A were added to the basal medium as controls. *Complement fixation tests* were attempted using the various serum components as antibody in order to substantiate serologically the role of complement in the inhibition of growth of PPLO. Antigens were prepared for only two strains, 07 and 60, by sedimenting the organisms from 2-day-old 150 ml broth cultures. The medium employed was Bacto-heart infusion broth with added 1% fraction A derived from bovine serum. The sedimented cells were washed in saline solution and resuspended in 10 ml of a 1:30 dilution of guinea-pig complement (Lyovac). The *fraction of bovine serum* separating at 34% saturation with ammonium sulfate and used in the concentration found in the original serum served as antibody in one case. Solutions of bovine alpha-, beta-, and gamma-globulins were made in saline solution in concentrations corresponding to that found in the original serum. These concentrations are as follows: alpha-globulin 0.84 g %, beta-globulin 0.78 g %, and gamma-globulin 0.66 g %. These various serum components employed as antibody were heated at 56°C for 30 minutes. The *hemolytic system* consisted of a 1.5% suspension of washed sheep red blood cells which were sensitized by rabbit anti-sheep red blood cell serum. To 0.5 ml *amounts of varying dilutions* of the serum

TABLE I. Inhibitory Effect on Growth of Pleuropneumonia-Like Organisms of Various Ammonium Sulfate Fractions of Bovine Serum without Added Complement.

Serum fraction	Mean colony diameters in μ Strain 07	09	48	60
0- 34% saturation	0*	0	0	0
34- 40% ”	0	0	0	0
40- 50% ”	37.9	23.3	0	0
50- 60% ”	43.8	63.0	23.4	15.8
60-100% ” (Fraction A)	105.4	74.0	107.5	121.2
Dialyzed serum	23.9	26.0	0	0
Normal ”	25.4	15.8	0	0

* Indicates that no growth occurred.

fractions ranging from 1:6 to 1:40 were added 0.5 ml of the antigens suspended in complement. After mixing and allowing to stand overnight in the refrigerator, the sensitized red blood cells were added. The tubes were read after 30 minutes incubation in a 37°C water bath. Hemolytic, red blood cell, antigen, and anticomplementary serum controls were also run.

Results. The inhibitory effect, measured as decrease in mean colony diameters, of the various ammonium sulfate fractions of bovine serum on the four test strains is shown in Table I. It can be seen that the inhibiting component separates with the globulin fractions when precipitated with ammonium sulfate. The inhibitor appears to be scattered throughout the globulin fractions. Dialysis does not remove the inhibitor. To further substantiate the protein nature of the inhibitory component in bovine serum the fraction separating upon 34% saturation with ammonium sulfate was subjected to heat, acid, alkali, and enzyme hydrolysis. Portions of this fraction containing a high concentration of growth inhibitor were heated in a boiling water bath for 10 minutes in N HCl, N NaOH, and at pH 7.0. The neutralized hydrolysates exhibited no inhibitory activity against the 4 strains of PPLO while the unheated control remained inhibitory. Digestion with pepsin and papain under optimal conditions for two hours altered the inhibitor enough that only slight inhibition was noted for the 2 less susceptible strains. The 2 highly susceptible strains were completely inhibited by the 2 enzymatic digests.

Further definition of the serum component inhibitory for PPLO was attempted with the ethanol fractions of bovine plasma containing as their major components the alpha-, beta-, and gamma-globulins, respectively. The addition of amounts of these various globulins equivalent to that found in a final concentration in the basal medium of 30% whole serum, as described previously, failed to inhibit the growth of PPLO. The higher degree of purification of the ethanol globulin fractions as compared with the crude ammonium sulfate fractions had apparently eliminated either the inhibitory component or some factor necessary for the inhibition. In a further attempt to associate the inhibitory activity of whole bovine serum to one of its globulin fractions, guinea pig complement (Lyovac, Sharp and Dohme) was added to the basal medium as an adjuvant to the various globulin fractions.

The effect of the addition of complement to the various preparations is shown in Table II. Inactivated bovine serum exhibited no inhibitory activity for strains 07 and 09 but partially inhibited the growth of the 2 highly susceptible strains, 48 and 60. The addition of complement to the inactivated serum restored the inhibitory activity to some extent. The 3 globulin fractions alone do not inhibit growth but, in the presence of complement, alpha-globulin markedly inhibits growth of PPLO. The inhibitory component is concentrated in

TABLE II. Inhibitory Effect on Growth of Pleuropneumonia-Like Organisms of Various Fractions of Bovine Plasma in Presence of Complement.

Plasma fraction	Mean colony diameters in μ Strain 07	09	48	60
Normal serum	26.0	19.2	0*	0
Inactivated serum	138.2	112.2	30.5	47.9
Inactivated serum + complement	80.7	46.6	17.8	0
α-globulin + complement	28.8	20.6	0	0
β-globulin + complement	64.4	58.8	57.5	47.9
γ-globulin + complement	64.4	58.8	56.2	39.7
Complement	63.0	54.8	39.7	35.6
Fraction A	131.8	117.9	71.2	71.2

* Indicates that no growth occurred.

TABLE III. Complement-Fixation Titers of Various Components of Bovine Plasma in Presence of Human Strains of PPLO.

Antigen	Plasma component 0–34	α-globulin	β-globulin	γ-globulin
07	1:5*	1:6	0	0
60	1:10	1:6	0	0

* Titer represents 50% fixation of complement.

the alpha-globulin fraction since the inhibition of growth caused by the beta- and gamma-globulins was not significantly different from that caused by complement alone. Complement in the form of normal guinea pig serum is inhibitory. As was mentioned above, guinea pig serum is one of the mammalian sera which are known to inhibit the growth of PPLO.

The presence of the growth inhibitor in the globulin fractions and its lability to heat, acid, alkali, and enzymes suggests an antibody-like nature. Experiments were run to determine if the inhibitor would fix complement in the presence of the organisms.

Table III lists the complement-fixation titers of the various bovine serum components in the presence of human strains of PPLO. The 34% ammonium sulfate fraction and alpha-globulin fixed complement in the presence of PPLO but the beta- and gamma-globulins failed to do so. Titration of the fractions used as antibody failed to reveal any anti-complementary activity. Hemolytic and antigen controls likewise exhibited complete hemolysis.

Since the alpha-globulin in the presence of complement causes inhibition of growth and in the presence of cells of PPLO fixes complement, it appears that the inhibitor found in whole serum is antibody-like in nature and is associated with the alpha-globulin fraction. That the ammonium sulfate fractions will inhibit growth without the addition of complement may be explained by the possibility that some complement or fraction of complement may be sedimented along with the other proteins during the crude fractionation.

Discussion. The antibody-like nature of the growth inhibitor for PPLO found in certain mammalian sera is evident from its protein nature, the requirement of complement for the initiation of growth inhibition, and the fixation of complement in low titer in the presence of the organisms. The partial destruction of the inhibitory component by enzymatic digest is in accord with the action of proteolytic enzymes on antibody for short periods of time. The inhibition of growth by the alpha-globulin in the presence of complement is possibly brought about by lysis of many of the organisms in the inoculum as well as those being produced during colony formation. There may possibly be a component of complement or of the guinea pig serum which is exerting an additive effect.

Beveridge, Campbell, and Lind(6) found a high percentage of normal human sera fixed complement in the presence of human strains of PPLO. It appears that only the sera of species presently known to be susceptible to infections with PPLO are inhibitory. Increasing the amount of toxic serum in the medium brings about greater inhibition of growth. It is probable then that a low antibody titer exists in most susceptible individuals and is indicative of previous exposure to PPLO. From the information obtained from these experiments, complement fixation would appear to warrant further investigation as a method for the diagnosis of infections caused by PPLO. The detection of circulating antibody-like substances in individuals might supply important information on the prevalence of these organisms.

Summary. Bovine alpha-globulin (bovine plasma fraction IV, Armour) in the presence of guinea pig complement inhibits the growth of the pleuropneumonia-like organisms. Heat-inactivated bovine serum is slightly inhibitory but the alpha-, beta-, and gamma-globulins are non-inhibitory in the absence of complement. Complement by itself does not inhibit growth as extensively as when in the presence of globulin. Alpha-globulin in the presence of the organisms fixes complement in low titer.

We wish to express our appreciation to Armour and Co. for supplies of bovine plasma fractions III-I and IV.

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Plasma Thromboplastin Component (PTC) Potency of Plasma Fractions.* (20268)

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(Introduced by G. S. Gordan.)

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In plasma thromboplastin component (PTC) deficiency disease, little or no shortening of the prolonged blood coagulation time or improvement in deficient prothrombin utilization follows the *in vivo* or *in vitro* addition of fraction I of Cohn(1,2). Other differential characteristics of the anti-hemophilic factor (AHF) and PTC are shown in Table I. A purified preparation of PTC, free of AHF, prothrombin and prothrombin conversion accelerators has been made by acidification of normal serum, adsorption of the serum with barium sulfate, and elution of the PTC from the barium sulfate with sodium citrate(2). This preparation is highly potent *in vitro*, but is unsuitable for intravenous administration in the human subject. The present investigation was conducted in an effort to find a potent PTC preparation suitable for intravenous administration among the fractions precipitated from plasma by varying the alcohol concentration and pH at low temperatures according to Cohn's methods(3).

TABLE I. Differential Characteristics of Plasma Thromboplastin Component (PTC) and Anti-Hemophilic Factor (AHF).

Source of material	PTC	AHF
Normal serum	P*	A*
Hemophilic plasma	P	A
PTC deficient plasma	A	P
45-50% saturated $(NH_4)_2SO_4$ fraction of normal plasma	P	A
0-33% saturated $(NH_4)_2SO_4$ fraction of normal plasma	A	P
Pseudoglobulin-albumin fraction of normal serum†	P	A
$BaSO_4$ adsorbed normal plasma	A	P
Citrate eluate from $BaSO_4$ which was mixed with normal plasma or serum	P	A

* P = Present; A = Absent.

† Supernate after removal of euglobulin by dilution and acidification.

TABLE II. *In Vitro* Effect of Plasma Fractions on Residual Serum Prothrombin in PTC Deficiency.

Substance added (0.1 cc to 2. cc of patient's blood)	Concentration, mg/cc*	Residual serum prothrombin† (units/cc)
0.32% sodium citrate in 0.9% NaCl		180
Normal plasma		<10
Fraction I	3.80	105
II	5.10	255
II + III	18.50	<10
IV	9.52	15
IV-1	4.09	<10
IV-4	5.43	100
V	29.8	200

* Each fraction was reconstituted to its original plasma concentration and dialysed against 0.32% sodium citrate in 0.9% NaCl overnight. The dialysis fluid was then changed and the fractions dialysed another 4 hr.

† Two-stage method of Ware and Seegers(4), after incubation of blood for 1 hr at 37°C.

Results. The PTC potency of the various fractions was determined by testing their

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† Major, M. C., U. S. Air Force.

TABLE III. Effect on Coagulation Time and Residual Serum Prothrombin of Addition of Fraction IV* to the Blood in Hemophilia and PTC Deficiency.

Substance added (0.1 cc to 2. cc of patient's blood)	Coagulation time† (min.) Hemophilia	PTC deficiency	Residual serum prothrombin‡ (% of normal) Hemophilia	PTC deficiency
0.9% NaCl	540	22	100	100
Undiluted fraction IV	35	13	100	10
1:10 fraction IV	—	13	—	14
1:100 ”	—	13	—	66
1:1000 ”	—	16	—	43

* Containing 6.5% protein.
† Done in duplicate in 13 mm tubes at 37°C.
‡ Two-stage method of Ware and Seegers(4), after incubation of blood for 1 hr at 37°C.

effect *in vitro* on the PTC deficient patient's defective prothrombin utilization. The PTC potency of fractions II + III, IV and IV-1 was high, that of I and IV-4 slight, and that of II and V none (Table II). When fraction III was separated from fraction II + III, it was found to contain thrombin, and therefore could not be assayed separately for PTC nor administered intravenously to the patient. None of the other fractions contained thrombin, as shown by the failure of coagulation to occur when 0.05 cc of the fraction was added to 0.5 cc of bovine fibrinogen (Armour) and allowed to stand at room temperature for 4 hours. Fraction IV-1, although potent, was not sufficiently soluble for intravenous use.

It is obvious from the *in vitro* tests that either fraction II + III or fraction IV might be suitable for intravenous administration, but since fraction II + III is used in the preparation of immune globulin, whereas fraction IV is discarded, it was decided to limit the *in vivo* investigations to fraction IV.

A preparation of fraction IV for intravenous use was made as follows: an 11% concentration of the fresh fraction was mixed in a Waring blendor, passed through a sterilizing porcelain filter, and frozen. After filtration it contained 9.2% total solids, 6.5% protein, 190 units of prothrombin per cc(4), and 69% of the proconvertin of an equivalent volume of plasma(5). *In vitro* tests with this preparation showed it to be active when added in a proportion of 1:20 of a 1:1000 dilution to the patient's blood (Table III). Tests for sterility, pyrogenicity, toxicity, and vasodepressor effects in rabbits were negative. No untoward effects on pulse, blood pressure, temperature or respiration were observed after intravenous administration of the fraction to a PTC deficient patient. Although the concentrated solution produced some shortening of the whole blood clotting time in hemophilic blood, it had no effect on prothrombin utilization. The results of two successive doses of 85 cc each of the fraction given intravenously to a PTC deficient patient at weekly intervals are shown in Table IV. The effect of each dose, derived from approximately 550 cc of plasma, was roughly comparable to that of 250 cc of plasma (Table V). Fraction IV therefore appears to contain approximately one-half of the PTC potency of plasma. The best preparations of fraction I contain only

TABLE IV. Effect on Coagulation Time and Residual Serum Prothrombin of Intravenous Administration of Fraction IV* in PTC Deficiency.

	Coagulation time,† min.	Residual serum prothrombin,‡ % of normal
Before 1st intrav. admin. of 85 cc of fraction IV	240	100
15 min. after 1st dose	11	31
3 hr ” ” ”	22	62
24 hr ” ” ”	18	57
2 days ” ” ”	22	56
4 days ” ” ”	21	61
7 days ” ” ” Before 2nd intrav. admin. of 85 cc of fraction IV	22	100
15 min. after 2nd dose	12	17
4 hr ” ” ”	12	24
24 hr ” ” ”	16	45
4 days ” ” ”	23	88

* Containing 6.5% protein.
† Done in duplicate in 13 mm tubes at 37°C.
‡ Two-stage method of Ware and Seegers(4), after incubation of blood for 1 hr at 37°C.

TABLE V. Comparative Effects of Intravenous Administration of 250 cc of Normal Plasma and 85 cc of Fraction IV* in PTC Deficiency.

	Coagulation time,† min.		Residual serum prothrombin,‡ units/cc	
	Plasma	Fraction IV	Plasma	Fraction IV
Before	>120	>120	260	260
15 min. after	15	11	85	82
4 days ”	17	21	134	164
7 ” ”	16	22	220	260

* Containing 6.5% protein.

† Done in duplicate in 13 mm tubes at 37°C.

‡ Two-stage method of Ware and Seegers(4), after incubation of blood for 1 hr at 37°C.

about one-tenth of the AHF potency of plasma(6). Fraction IV is present in the plasma in a concentration of approximately 1 g per 100 cc, while fraction I is present in a concentration of only 0.38 g per 100 cc. It would, therefore, require almost twice the volume of an equivalent protein concentration of fraction I derived from 5 times the original quantity of plasma to produce similar results in hemophilia.

Conclusions. 1. The PTC factor is concentrated in fractions III and IV-1 of the plasma proteins. Small quantities are found in fractions I and IV-4. None is found in fractions II and V. 2. Since fraction III contains thrombin and fraction IV-1 is poorly soluble, neither is suitable for intravenous administration. 3. The intravenous administration of fraction IV produced marked shortening of the whole blood coagulation time and reduction in residual serum prothrombin in PTC deficiency. The results appear superior to those which can be achieved by the intravenous administration of fraction I in hemophilia.

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Metabolic Products of Cholesterol in Bile and Feces of Rat.* Steroids and Bile Acids. (20269)

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The transformation of cholesterol into cholic acid was first demonstrated in the dog with deuterated cholesterol(1). The same reaction has recently been shown to occur in the rat with cholesterol-4-C^{14}(2,3). In the latter work we further showed that cholic acid was the main excretory end product of cholesterol metabolism constituting more than 80% of the labelled acidic products of the bile. This agrees with observations(4,5) that most of the isotope of administered cholesterol-4-C^{14} that was excreted came via the bile and feces as acidic products and that only very small amounts appeared in the expired carbon dioxide. That the labelled acidic products in the bile and feces behaved like bile acids in fractionations has subsequently been shown in the same laboratory(6).

In this paper some further results will be presented on the fractionation of the acidic products in bile and feces of rats that had received cholesterol-4-C^{14} by intraperitoneal administration.

Experimental. One mg of cholesterol-4-C^{14}

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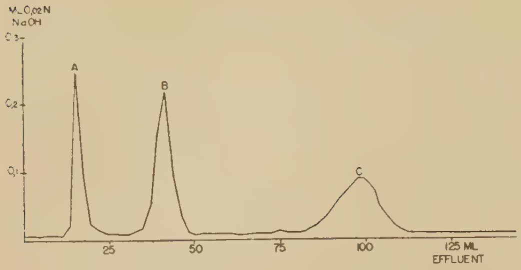

FIG. 1. Chromatography of taurocholic (A), glycocholic (B) and cholic acid (C). Stationary phase: 4 ml of octanolchloroform 50-50 on 4.5 g hydrophobic Supercel. Descending phase: 50% aqueous methanol.

containing 5 μc C^{14} was administered intraperitoneally to 200 g white rats in an aqueous colloidal solution. It was prepared by dissolving 1 mg cholesterol-4-C^{14} in 0.1 ml of hot ethanol. To this solution was added 2 ml of hot saline containing 0.12 mg of sodium palmitate; the mixture was heated at 90°C for about half a minute when a practically water clear solution was obtained. It has been found that cholesterol administered in this way was rapidly absorbed from the abdominal cavity and distributed in the body (10). After the injection the rats were kept in metabolic cages and the feces collected daily in ethanol. When the bile was to be collected, the rats were kept in a restraining cage after the introduction of a polythene cannula into the bile duct and the bile was collected directly into ethanol(3). The animals had free access to their normal stock diet and water or in the latter case 0.9% sodium chloride solution.

Fractionation of unhydrolyzed bile. The precipitated mucoids were filtered off and the filtrate taken to dryness *in vacuo*. The residue was dissolved in a small volume of water, acidified to pH 1 with hydrochloric acid and extracted 3 times with 2 volumes of butanol. The combined butanol extracts were then washed with distilled water until free of hydrochloric acid—all wash-waters passing a second butanol phase. The combined butanol phases contained about 95% of the total activity. After evaporation to dryness *in vacuo* the residue was subjected to reversed-phase partition chromatography(7,8). After titrating each chromatographic fraction it was plated on copper or aluminum planchets for determination of the isotope content. For reference a chromatographic separation of taurocholic, glycocholic and free cholic acid is illustrated in Fig. 1(8). The chromatography of material from the butanol extract of the bile from a rat that had received labelled cholesterol is shown in Fig. 2. The stationary phase that was washed out with chloroform contained about 15% of the original activity as neutral compounds. We have earlier shown that the influence of the taurine group is so pronounced that even the taurine conjugate of the monosubstituted lithocholic acid leaves the column before glycocholic acid under these conditions(8). It is therefore clear that practically all activity in acidic compounds was contained in compounds that behaved like taurine conjugates.

Fractionation after alkaline hydrolysis. The fractions containing the activity 10-25 effluent

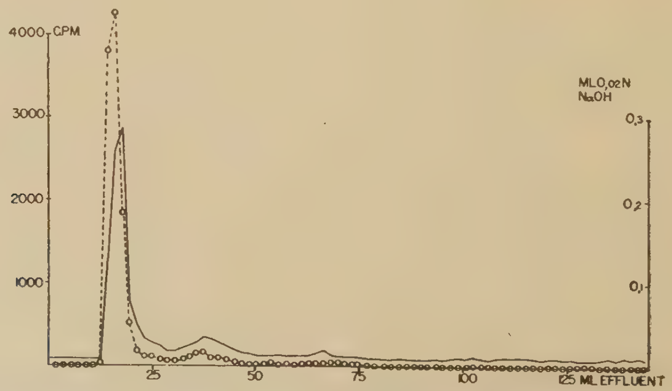

FIG. 2. Chromatography of butanol extract of unhydrolysed bile excreted on 8th day after intraperitoneal administration of cholesterol-4-C^{14}. Conditions: Fig. 1.

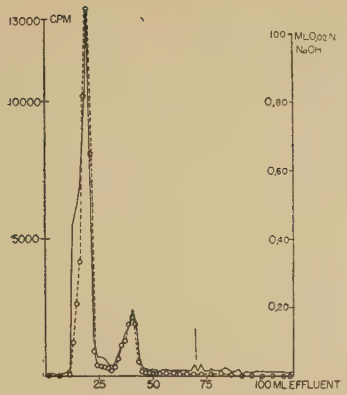

FIG. 3. Chromatography of hydrolysate of active fractions in Fig. 2 (10-25 ml effluent). Stationary phase: 4 ml of chloroform-heptane 9:1 on 4.5 g hydrophobic Supercel. Descending phase: 60% aqueous methanol.

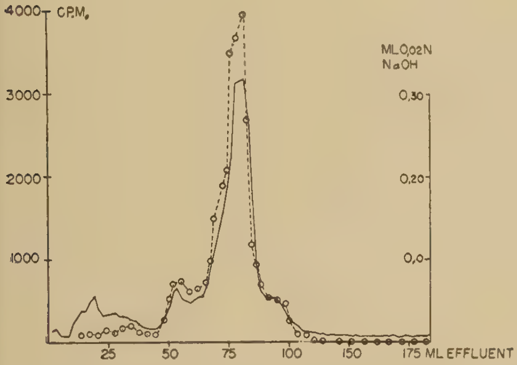

FIG. 4. Chromatography of material from cholic acid band (Fig. 3) (10-25 ml effluent). Stationary phase: 4 ml octanol-chloroform-heptane 45-45-10 on 4.5 g hydrophobic Supercel. Descending phase: 45% aqueous methanol.

(Fig. 2) were combined in an aqueous solution and hydrolyzed in one normal sodium hydroxide at 140° for 3 hours. The solution was then acidified with hydrochloric acid, extracted with ethyl ether and the extracts combined, washed with water and evaporated to dryness. Chromatography of these acids gave the results shown in Fig. 3, *i.e.*, a curve similar to normal rat bile with a large band in the cholic acid region followed by a smaller one in the region where dihydroxycholanic acids appear(7,9). The second band present in normal rat bile has been found to be chenodesoxycholic acid(11). The activity is also present as chenodesoxycholic acid as dilution with inactive chenodesoxycholic acid and recrystallization has shown that the specific activity remained constant. With isotope dilution it has already been shown that the main isotope-containing component of the first band is cholic acid(3). When this fraction is re-run with a more polar stationary phase a number of minor active fractions was found to appear, one just before and one just after the main part of the activity contained in cholic acid (Fig. 4). The labelled compounds in these bands have not yet been identified. It should be noted that bile secreted during the first day after the bile duct cannulation is not present in this material, *i.e.*, bile acids that have reached the intestine and have been in contact with the intestinal flora are not included.

Fractionation of activity in feces. After intraperitoneal administration of the colloidal solution of cholesterol-4-C^{14} the feces were collected in ethanol. The feces from every 24 hours were homogenized and extracted 3 times with boiling 90% ethanol. The isotope content was determined on a small aliquot of the combined filtrates. The remainder was evaporated to dryness *in vacuo*. The residue was dissolved in 25 ml of 0.2 M sodium carbonate and this solution was extracted 3 times with a large volume of ether. The ether extracts were combined, washed with water and evaporated to dryness.

The aqueous phase was then acidified to pH 1 with hydrochloric acid and extracted with butanol. The combined butanol extracts were washed with water until free of hydrochloric acid. The residue from the butanol extracts was distributed between equal volumes (30 ml) of 70% ethanol and light petroleum by a 3-stage counter-current extraction. The residue from the combined aqueous phases was then chromatographed as shown in Fig. 5 (7,8) with a solvent system suitable for separation of conjugated bile acids (Fig. 1 and 2). The material that stayed in the stationary phase was eluted with chloroform and then subjected to a new separation, as shown in Fig. 6, in a system suitable for the separation of unconjugated bile acids.

From a comparison of Fig. 5 and 6 with Fig. 1 and 2 it is noticed that whereas in the bile practically all the labelled acidic com-

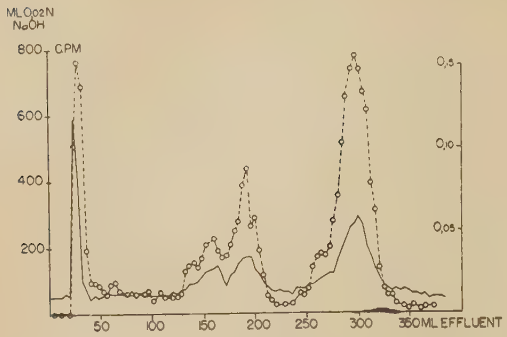

FIG. 5. Fecal acids from 7th day after intraperitoneal administration of cholesterol-4-C^{14}. Conditions: phases equal to Fig. 2. Column: 9 g hydrophobic Supercel.

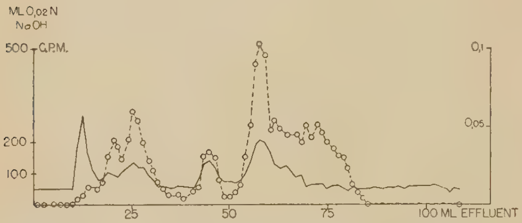

FIG. 6. Fecal acids: Material remaining in stationary phase Fig. 5 eluted with chloroform and re-run under conditions identical with those in Fig. 3.

pounds are present as taurine conjugates only a small part is present in this form in the feces.

However, it is not only a simple splitting of the peptide bond of the conjugates present in the bile that has taken place in the intestine. No activity is found at the place where free cholic acid appears (100 ml effluent). Instead a number of new bands are present of which 3 major ones that have been observed regularly are seen in Fig. 5—two eluted earlier and the largest one later than cholic acid. The less hydrophilic acids that stay in the stationary phase in the run shown in Fig. 5 were eluted with chloroform and rechromatographed as shown in Fig. 6. They apparently also consist of a complicated mixture of presumably unconjugated bile acids.

Thus the taurocholic acid that is the main labelled excretory product in the rat bile has not only been split but the cholic acid has been further modified in various ways by the intestinal microorganisms.

This is of course in accord with a number of old observations that the bile acids present in bile cannot be found in the feces. As expected some of the compounds have been found to contain ketonic groups.

This is also in accord with the findings of Schmidt, Hughes, *et al.*(12) on the metabolism of cholic acid in the guinea pig and their demonstration that this bile acid was transformed into dehydrocholic acid by *Alcaligenes faecalis.*

Summary. 1. After administration of cholesterol-4-C^{14} to rats the main excretory products in the bile are cholic and chenodesoxycholic acid present as taurine conjugates. 2. The labelled material present in feces has been fractionated. It has been found that most of the conjugate has been split. No unchanged cholic acid seems to be left in the feces. A series of different labelled products have been separated some at least of which contain ketonic groups.

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Effect of Fever on Serum Polysaccharide Level. (20270)

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Previous work has shown that the serum polysaccharide level of dogs becomes elevated in experimental inflammation caused by infection with bacteria, intrapleural or intramuscular injection of turpentine, intraperitoneal injection of a talc suspension, or surgical operation(1) and after epidermal thermal injury(2). It was noted that this elevation occurred both in the presence and absence of fever. This observation raised the question as to whether fever had any direct connection with the elevation of serum polysaccharide. In order to answer this question, experimental fever was induced in normal dogs by various means and the serum polysaccharides determined serially. As it appeared feasible that the polysaccharide content of one serum protein fraction might increase relative to the others, studies of these fractions were also made serially.

Methods. In this study nonglucosamine polysaccharide was determined by the tryptophan method(3) and serum was fractionated as previously described(4) by precipitation with 21.6% and 26.0% sodium sulfate. The fraction not precipitated by 26.0% sodium sulfate was assumed to be largely albumin and is designated in this paper as the albumin fraction, while that not precipitated by 21.6% was assumed to be combined albumin and pseudoglobin (α-globulin). Mucoprotein polysaccharide was determined by the tryptophan method after isolation of the mucoprotein fraction by the method of Winzler and Smyth(5). Fever was induced in one set of seven dogs (group 1) by muzzling the animals

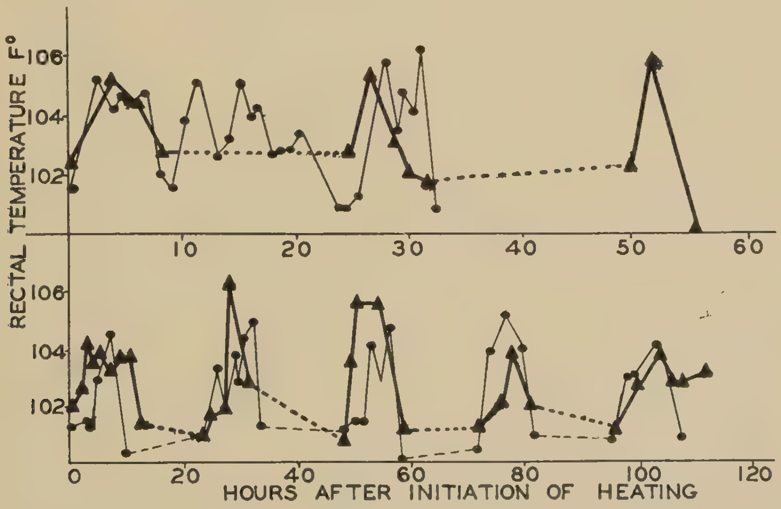

FIG. 1. Typical temperature curves of dogs in which fever was induced by various means. Upper curves: ●——● group 1, heated by external means; ▲——▲, group 3 given pyromen inj. Lower curves: ●——● group 2, heated by external means, ▲——▲ group 4 given pyromen inj.

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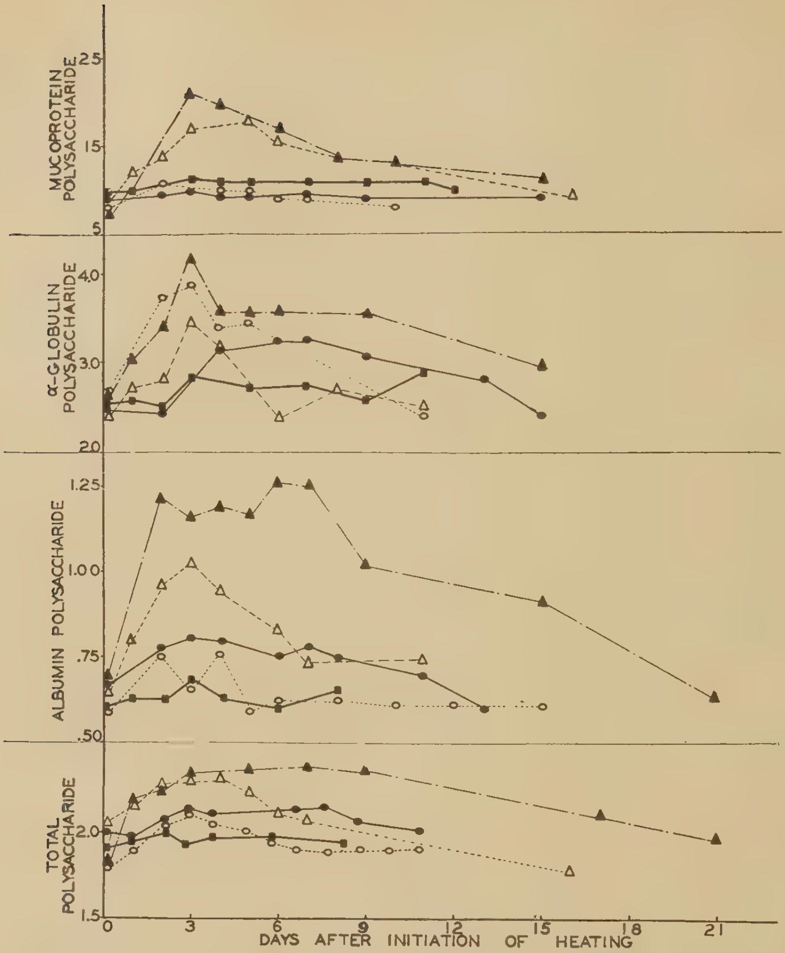

FIG. 2. Effect of fever on the nonglucosamine polysaccharides of serum protein. The different groups are denoted graphically: (1) ○- - - -○ heated externally 32-48 hr, (2) ●——● heated externally for 8 hr intervals for 5 days, (3) △- - - -△ given pyromen inj. for 8 hr intervals for 3 days, (4) ▲——▲ given pyromen inj. for 8 hr intervals for 5 days, and (5) ■——■ control group. Total polysaccharide is denoted as % of the serum protein, "albumin" polysaccharide as % of the albumin protein, "α-globulin" polysaccharide as % of α-globulin, and mucoprotein polysaccharide as mg of hexose per 100 ml of serum.

and wrapping them in a blanket containing a heat pad. Considerable elevations of temperature were induced and maintained by this method as shown in Fig. 1. At 3-4-hour intervals the animals were unmuzzled and allowed to drink. This heating process was continued for 24 to 48 hours. Rectal temperatures were taken every two hours. A second set of four dogs (group 2) were heated by the same procedure for periods of 8 hours daily for 5 days. Six dogs (group 3) were subjected to repeated intravenous injections of the pyrogenic substance pyromen[†] in order to maintain a fever. Injections were continued for a period of 2 days. Rectal temperatures were taken at two-hour intervals. A fourth set of dogs (group 4) was subjected to pyromen injections in order to maintain elevated temperatures for 8-hour intervals over a period of 5 days.

Results. The average data for each set of dogs are summarized in Fig. 2. The data for each day following heating were compared statistically with the data obtained before

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[†] Supplied by Baxter Laboratories, Morton Grove, Ill., through the courtesy of Dr. L. G. Ginger and Dr. W. F. Windle.

TABLE I. Summary of Statistical Comparisons.

		Days on which significant elevations occurred in polysaccharide of			
Group*	No. of animals	Total protein	Albumin	Pseudo-globulin	Mucoprotein
1	7	4†	0	0	0
2	4	3†	0	3†	0
3	6	2†	2,§ 3,§ 5†	2§	5§
4	3	1,† 2,‡ 3,§ 15§	1,‡ 2,‡ 5,‡ 7†	2,† 3,† 5§	1,‡ 2,‡ 3,§ 7,§ 9‡

* Groups 1 and 2 were heated by external means, Group 1 for 2-3 days, Group 2 in 8 hr periods each day for 5 days. Group 3 and 4 heated by inj. of pyromen, Group 3 for 2-3 days, Group 4 in 8 hr periods each day for 5 days.

† Significant at 5% level. ‡ Significant at 2% level. § Significant at 1% level.

treatment. The averages were compared by the conventional "t" test, with results as given in Table I.

An elevation in total nonglucosamine polysaccharide was obtained in all groups of animals after heating. Response of individual animals of group 1 and 2, in which fever was induced by external heating, however, was variable, some individuals experiencing little elevation. Response of animals given pyromen was much more consistent. Noticeable elevations of total polysaccharide occurred within 24 hours, but significant elevations did not occur, except in group 4, until the second, third or fourth day. An elevation of the polysaccharide content of the pseudoglobulin usually occurred whenever an elevation of the total polysaccharide occurred. Due to variations between dogs, no significant increase of this factor was noted in group 1, although a considerable average increase was found as shown in Fig. 1. The maximum elevation of pseudoglobulin polysaccharide occurred on about the third or fourth day after initiation of treatment. A significant elevation of albumin polysaccharide was noted in both groups of dogs given pyromen injections. This elevation occurred somewhat before the elevation of the polysaccharide of the pseudoglobulin fraction, being significantly elevated on the first day after the first injection in group 4 and the second day in group 3. This elevation of albumin polysaccharide was found to be due in every instance to an increase of mucoprotein polysaccharide. In contrast to group 3 and 4 in which fever was induced by injections of pyromen, no significant increase in albumin or mucoprotein polysaccharide was noted in the animals in which fever was induced by external means.

Discussion. The elevations of total, pseudoglobulin, and mucoprotein polysaccharide levels of animals subjected to other stress, *i.e.*, epidermal thermal injury(2), were considerably greater and more consistent than those found in this study. Fever caused by bacterial pyrogens in the animal body may be expected to affect both the mucoprotein and pseudoglobulin fraction. In contrast to cancer patients(4) or pregnant women(6), the polysaccharide of the albumin fraction, after correction for the mucoprotein, is not significantly elevated following fever.

Summary and conclusions. Experimental fever in dogs caused by external heating or by injection of a pyrogenic substance resulted in a significant elevation of total serum nonglucosamine polysaccharide. An elevation of the polysaccharide content of the pseudoglobulin was noted in all animals, while an elevation of mucoprotein was noted only in the animals subjected to the injections of the pyrogenic substance, pyromen.

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Effect of Electroshock and Electronarcosis on Intraocular Ovarian and Endometrial Transplants.* (20271)

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Women receiving electroshock (ES) and electronarcosis (EN), frequently have a temporary suspension of menstruation during and following intensive treatment(1). For this no adequate explanation has been offered. Treatments given a week apart do not have this effect. Clarification of these facts may aid our understanding of what takes place during electroconvulsive treatment(ECT).

Various reports have appeared indicating that electrical current application to animals resulted in hypophyseal stimulation(2-6). Marshall and Verney(7) reported that electrical currents applied to the head of rabbits can induce ovulation. This does not occur when the stalk of the pituitary is severed. That EN affects hypophyseal activity is indicated in the studies of Ellis and Wiersma(3) reporting hypertrophy of the thyroid, gonads, and adrenals.

Method. We removed the ovaries of rabbits and transplanted them to the anterior chamber of the eye. This makes it possible to follow ovarian developments and since the ovary is severed from its normal nerve connections, we can be certain of the hormonal nature of the stimulation of the ovaries by ECT. Endometrium was also introduced into the eye to serve as easily observable target tissue for the transplanted ovary. Twenty-eight adult female Dutch rabbits, averaging 4½ months of age, were studied in 4 groups. Prior to and during experimentation the animals were kept in individual cages to prevent pseudopregnancy. They were maintained at a temperature of 70°F, and were fed standard rabbit pellets. Water was given *ad libitum.* All 28 animals received intraocular ovarian implants, and 22 animals in addition received endometrial transplants, after the method of Benjamin, Belt, and Krichesky(8). The animals were castrated to obtain tissues for transplantation. Moreover, we observed that removal of the ovaries was necessary in order that pituitary hormones would reach the transplant. This differential absorption of endogenous hormone by the target gland *in situ* has been previously described(9). One ovary in each case was preserved in Bouin's fluid, as was also a uterine sample (subtotal hysterectomy), for later histological examinations. Frequent observations and drawings were made of the transplants. Occasional photographs were taken. At the conclusion of each series, the animals were sacrificed by intravenous injection of air. We fixed tissues (intraocular transplants, adrenals, pituitary, thyroid, and uterus) in Bouin's fluid and prepared them for later histological studies.

Treatment procedure. ES was induced with 60-cycle sinusoidal current applied to the head according to a procedure outlined for use with rabbits(10,11). The onset was of the glissando type(12). Since we were using a smaller breed of rabbit we employed an initial current of less intensity (40-45 ma) for 30 seconds during which the complete seizure pattern was "tripped" as indicated by the development of a strong extensor stretch. The current was cut at 30 seconds, and the grand mal seizure was ended with vigorous clonic contractions which in some cases were followed by strong walking movements. Respiration was resumed 30-40 seconds after the beginning of the extensor stretch as with human beings. EN diverges from ES after the first 30 seconds when the current instead of

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The ECT equipment used in this study is a standard treatment apparatus manufactured by the Electronicraft Company, Los Angeles, Calif. and specifically calibrated to allow for control of minute amounts of current.

being cut is dropped to 5 ma. Respiration, however, is resumed within 30-40 seconds after the beginning of the extensor stretch as with ES treatment. Then the current is raised to a point compatible with a quiet narcosis marked by good muscle tone, salivation, and a minimum of stridor so that there is deep regular breathing. The total duration of the EN treatment in this experiment was limited arbitrarily to 5 minutes. With few exceptions, upon termination of EN treatment the rabbit righted itself instantaneously with no cataleptic symptoms appearing. This is in direct contrast to the slow awakening in the ES-treated animals which pass through an intermediate stage of catalepsia.

Preliminary experiments. Preliminary experimentation with 12 animals suggested the following conclusions: 1. Both ES and EN treatment caused growth of the transplanted tissues. 2. ES-treated animals, however, would appear to require about twice as many treatments before growth was observed. 3. In non-castrates, no stimulation of ovarian transplants occurred following treatments; although transplanted endometrium responded. Furthermore, upon direct observation of the animals the ovaries and uteri showed evidence of stimulation.

Experiment. As a check upon these findings 16 additional animals were castrated and received intraocular transplants. After one week all 16 had viable implants. By the 17th day after castration and implantation we could see no evidence of growth. On the 18th day we applied EN to 8 animals, ES to 3, and did not treat the other 5. The treatment schedule approximated that given to human patients: one was given each day the first 7 days with the remainder spaced about every other day for a total of 12 treatments. Six of the EN-treated animals showed growth after 2 treatments and two required 3. Growth continued throughout the rest of the experiment. Two of the ES-treated animals showed growth after 6 treatments; one required 7 treatments. No growth was observed in the controls throughout the experiment.

Microscopic observations. Effect of EN on intraocular transplants. The ovaries at the time of transplantation were immature (translucent, small, few follicles with antra), and the uterus *in situ* was still infantile. Fifty-nine of the 62 transplants in all the EN-treated animals (including the 8 preliminary animals) remained viable. Definite hyperemia and activity were observable after the third EN treatment in 2 of the EN animals and after the second treatment in 6 of the animals. Occasionally bleeding into the anterior chamber was noted immediately following treatment. More frequently, in the ovar-

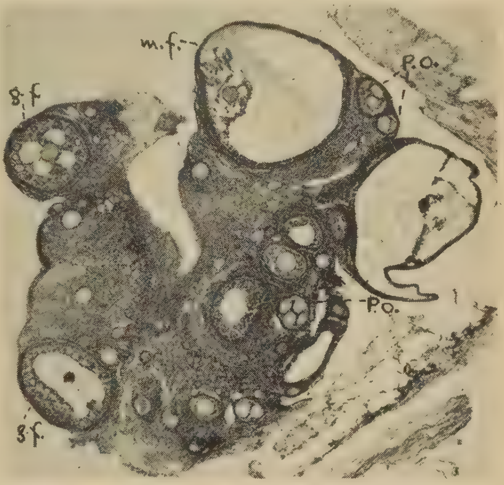

FIG. 1. Ovarian intraocular transplant of rabbit EN-8. Note growing follicles (g.f.) of varying sizes; polyova (P.O.) with definite nuclei; mature follicle (m.f.); numerous primary follicles; and some atretic follicles. No luteal tissue is present. H. and E. × 30.

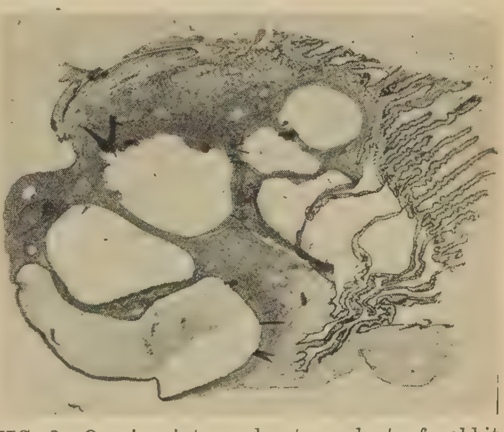

FIG. 2. Ovarian intraocular transplant of rabbit EN-4. Observe marked cystic dilation of follicles, compression of granulosa cell layers, cloudiness of follicular fluid when present, and extravasated blood. H. and E. × 15.

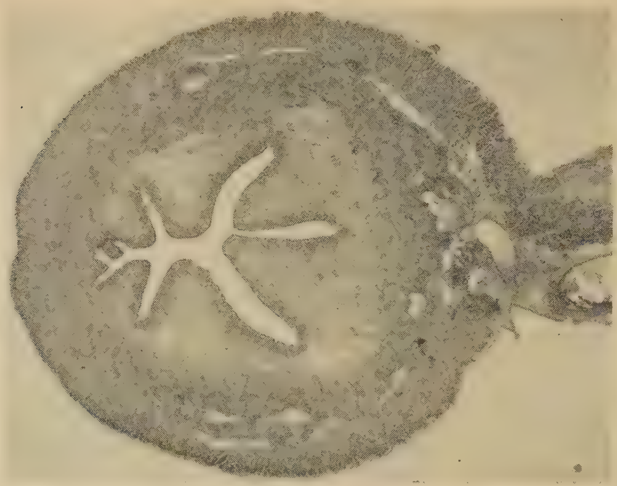

FIG. 3. Cross section of uterus of rabbit EN-1 removed at necropsy following 12 EN R_x, showing a typical estrus or proliferative condition. H. and E. × 30.

ian transplants, *blutpuncta* were seen. As a general rule, growth once initiated continued as treatments progressed. Vesicular follicles appeared and increased both in size and in number. Histological sections of ovarian transplants indicate definite stimulation and growth (Fig. 1, 2). Two patterns, or degrees of response, appeared. In one (Fig. 1), highly activated transplants include follicles in all stages of development from primordial to mature follicles. Strikingly, many multiple follicles occur. Inasmuch as polyovular follicles are not a common occurrence in the non-stimulated rabbit ovary, an excessive hypophyseal discharge of gonadotropins might have disrupted the complicated histogenic interrelationship described by Dawson(13) as existing between the germinal epithelium, oovocytes, and the initial layer of follicular cells. In the other pattern (Fig. 2), cystic follicles characteristic of a hyperstimulated ovary predominate.

Secretory activity of the intraocular ovarian transplants is evident in the appearance of the uterine tissue, both *in situ* and in the eye. Typical estrogen effects—glands with straight lumina, edematous stroma particularly in the deeper layers, well developed muscularis and serosa, and hyperemia throughout—are illustrated in a specimen removed at necropsy following 12 EN treatments (Fig. 3). The intraocular endometrium increase in area and hyperemia synchronously with the activation of the intraocular ovarian tissue(4). The proliferative property of endometrium is shown in the invasion of adjacent eye tissues by the epithelium as it covers the surface of the entire transplants and extends onto the iridial tissues(5). Frequently, it appears to undergo a metaplasia, as described by Allen and Priest(14), to a type resembling tubal epithelium. Luteinization, although infrequently

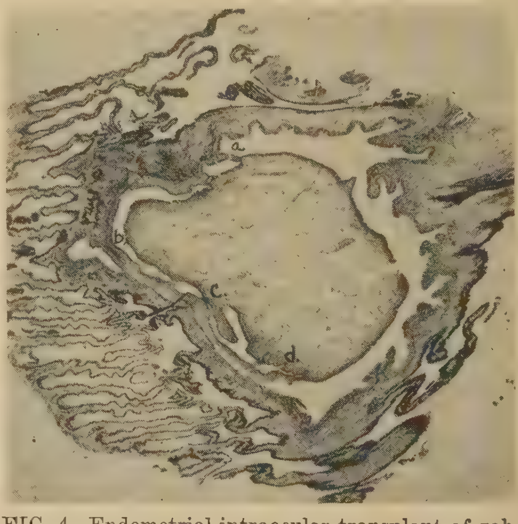

FIG. 4. Endometrial intraocular transplant of rabbit EN-5 (see Fig. 2). Size, organization, stromal nuclei dispersion, and extensive vascularization attest to estrogen stimulation of the transplant (compare with control, Fig. 7). Note complete coverage of transplant by the epithelium and its proliferation onto adjacent eye tissues at a, b, c, and d. H. and E. × 23.

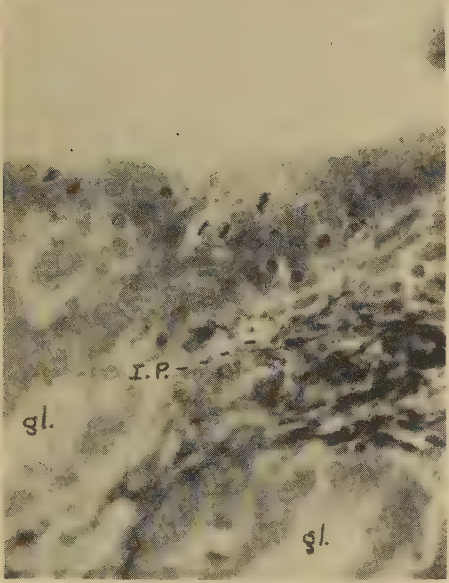

FIG. 5. High power of *c* in Fig. 4. Mitoses evident. Note hyperplasia, forming gland-like structures (gl.) in the iris tissue. Iridial pigment is present at I.P. H. and E. × 487.

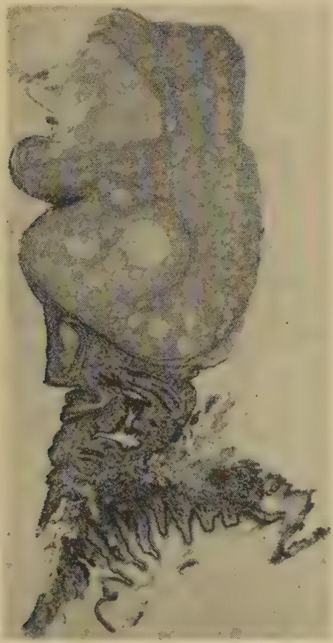

FIG. 6. Ovarian intraocular transplant of rabbit ES-1. Note numerous follicles, majority devoid of follicular cells, and many atretic follicles in early stages of development. H. and E. × 30.

observed, was seen only as very small clusters of polyhedral lipochrome cells, scattered occasionally through the ovarian stroma.

Effect of ES on intraocular transplants. The number studied (6, including the preliminary studies) was small, yet several interesting facts were noted. It was apparent that twice the number of ES treatments are necessary to activate the intraocular transplants as are required when EN treatment is used. Once initiated, the growth seemed to follow a slower rate and the transplants developed hyperemia more slowly. Ovarian intraocular tissue in such animals consisted chiefly of primordial follicles (Fig. 6), and only a few follicles showed antra development. In those animals in which growth approached that in the EN rabbits, the ovarian transplants became grossly cystic at the time of the 9th or 10th treatment. This would be followed by a definite decrease in hyperemia, and by the time of necropsy (3 days after final treatment) atrophy had occurred. Why these transplants behaved differently from those observed in the EN animals poses an interesting question and invites further study.

Intraocular transplants of non-treated animals. This group, in direct contrast to either of the other two, showed either typical castration atrophy of the endometrial intraocular

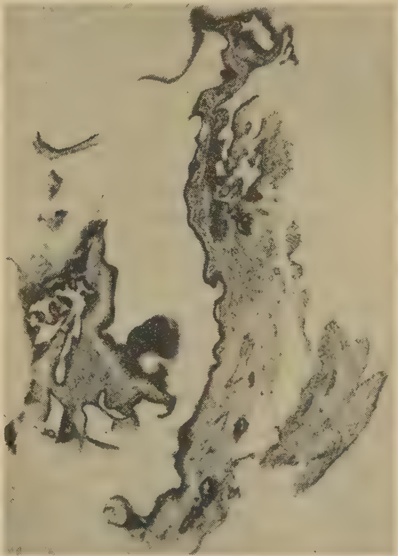

FIG. 7. Endometrial intraocular transplant of rabbit C-3 (control, receiving no shock R_x). Note disorganized, avascular, and slightly developed endometrium typical of a castrate. H. and E. × 30.

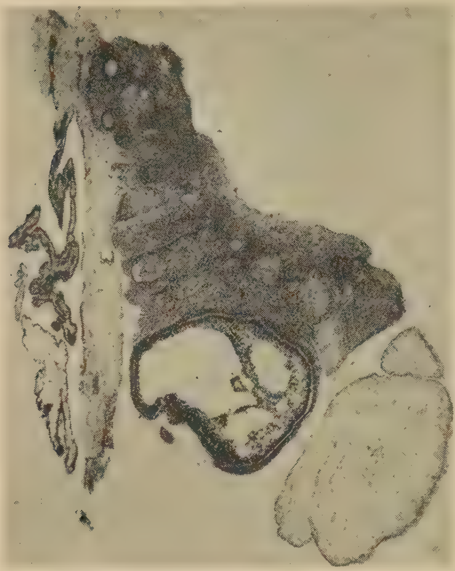

FIG. 8. Ovarian intraocular transplant of same animal (Fig. 7). Transplant is relatively small and consists chiefly of solidly packed stroma. Follicles present, other than the one Graafian, are very small or are undergoing atresia. H. and E. × 30.

transplants (Fig. 7) and ovarian transplants which contained solidly packed stroma and few follicles (Fig. 8) or endometrial transplants just barely maintained, displaying no progressive growth. In the latter, the accompanying ovarian transplants manifested little physiological activity, and the uteri *in situ* were slightly motile at necropsy; otherwise the uteri were smaller, avascular and completely flaccid.

Summary and conclusions. 1. Intraocular ovarian transplants of gonadectomized rabbits subjected to ECT show follicular stimulation. Twice as many ES treatments were required as EN to obtain growth. Follicles were observed in all stages of development, including both maturing and cystic. Hemorrhagic follicles occurred frequently but terminated in follicular atresia rather than ovulatory corpora lutea. The experimental animals gave good evidence of estrogen production by the ovarian intraocular transplants in the proliferative condition of both the endometrial transplants and the uteri *in situ*. (No attempt was made to exclude possible adrenal cortical activity.) This was in direct contrast to the lack of activation and even atrophy of transplants (and uteri *in situ*) in similarly prepared but non-treated controls. 2. It is known that menstruation may be prevented by maintenance of high estrogen levels. This sometimes occurs in the presence of cystic ovarian follicles as well as during continued administration of estrogens. 3. Continued follicular growth with resultant maintenance of high estrogen secretion may result from stimulation of the hypothalamic-pituitary neuro-humeral pathways by the electric current. Such a "constant estrus" condition might account for the interruption of the cyclic activity in women receiving ECT, as well as its resumption as treatments are spaced farther apart.

We are indebted to Dr. A. van Harreveld and Dr. C. A. G. Wiersma for their helpful assistance and advice during the course of these investigations. We gratefully acknowledge the assistance of Miss Ruth E. Estey in making histological preparations. Mr. G. H. Needham prepared the photomicrographs.

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Effect of Castration in Male Mice on *Schistosoma mansoni*. (20272)

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Schistosoma mansoni (Sambon, 1907) has received extensive study both clinically and in the laboratory, but only recently has attention been focused on the physiological aspects of the parasite. Investigators(1-3) have noted the variations which occur with unisexual and bisexual infections obtained by introducing different ratios of male and female cercariae. Niyamasena(4), Gönnert(5), and Vogel(6,7) have studied the genetic phases and the histological development of the male and female worms. Variations have been detected in the sexual apparatus of the parasite by Vogel(7), Short(8,9), Gönnert(5), Najim(10) and Buttner(11). In this respect Buttner has found changes in different hosts. Thus the question of the possible influence of hormones, both from the parasite and the host standpoint is implicated. Addis(12) made interesting studies on several sex hormones and their effect on the growth of certain cestodes in rats. In his results he indicated that testosterone was necessary for the growth of these parasites, in male rats. Since cestodes and trematodes are both in the same phylum (platyhelminthes), it was thought by the writer that a similar relationship might exist between this hormone and *S. mansoni*.

An attempt has been made to see if any morphological change occurs, specifically that of the sexual apparatus of *S. mansoni,* in castrated male albino mice. After careful study it may be stated that morphologically the male and female worms recovered appeared to show normal sexual development. The females produced normal eggs while the males produced normal spermatozoa. However, there was a marked difference in the number of worms recovered from the castrated as compared to the uncastrated control mice. Further investigations on the cause of this phenomenon are now being carried on.

Materials and methods. Male albino mice (Rockland RAP strain) were employed. Fifty-five mice were surgically totally castrated at 5-6 weeks of age. Fifteen uncastrated mice of the same age group were used as controls. The average weight of the animals was 17.2 g at the time of castration. They were maintained throughout their life in white porcelain pans 12 x 8 x 5 inches with a wire screen top containing a depressed feeder. Each pan contained 7 mice which were fed exclusively on Purina Laboratory Chow, and each pan contained a watering bottle. The castration was done in the following way: The mice were anesthetized with sodium pentobarbital (Abbott), the dosage being 0.1 mg per gram body weight. The animal was then strapped to a dissecting board and taped across the abdomen to prevent the testis from reverting up the inguinal canals. A vertical incision 1-2 mm was made medially through the scrotal septum and the tunicas, thus exposing both testes. Each was clamped, ligated and excised at the epididymis level resulting in total bilateral castration. The scrotum was then clamped and sutured using two stitches. Four weeks after recovery, the castrated and uncastrated control mice were infected with approximately 140 pooled cercariae per mouse, at The Tropical Disease Laboratory, National Institutes of Health, Bethesda, Maryland, through the courtesy of Dr. Willard H. Wright. The source of the cercariae was laboratory bred snails of the species *Australorbis glabratus* (Say). The method of infection used was that of Olivier and Stirewalt(13) in modification. *Eggs* in the feces of the mice were first observed on the 45th day after infection. Previous examination revealed no visible eggs. The methods of stool examination employed were by direct smear in 1% eosin solution and by the Mathieson and Stoll Acid-Ether Method(14). Both the experimental and control animals were sacrificed at random 63-68 days after infection. They were killed with ether and the worms were perfused from the liver and intestine by the method of Yolles

TABLE I. *Schistosoma mansoni,* Total Adult Count by Sex Recovered from Castrated and Uncastrated Control Mice 63-68 Days after Infection.

	Castrated mice (43)	Uncastrated control mice (13)
Total worms	999	442
Male	671	321
Female	328	121

TABLE II. *Schistosoma mansoni,* Mean Adult Count by Sex from Castrated and Uncastrated Control Male Mice 63-68 Days after Infection.

	Castrated mice (43)	Uncastrated control mice (13)
Total worms	23.23	34.00
Male	15.61	24.69
Female	7.63	9.31

et al.(15). The tissues were then carefully teased to see whether any worms remained embedded therein. The schistosomes were then separated by sex and counted with the aid of a dissecting microscope.

Results. A total of 1442 *S. mansoni* adults were collected from both groups of mice with 999 from 43 castrated mice as compared with 442 from 13 uncastrated controls. For the total male and female worm population in the castrated mice a mean of 23.23 worms per mouse resulted, while in the uncastrated control group a mean of 34.00 per mouse resulted, indicating a difference of 10.37. The median and mode corresponded closely and the statistical analysis was based upon a normal probability curve. The Student "t" test for significance indicated that this difference was highly significant, the t value being 4.14 at the 5% level.

What is most interesting is that further analysis of individual sex indicated a significant difference between the male worms only.

A total of 671 *males* were collected from the castrated group while the uncastrated controls produced 321. The mean adult count per mouse was 15.61 for the former group and 24.69 for the latter indicating a reduction of 9.09 schistosomes. As analysed by the Student test, the "t" value of 4.32 for this difference was, in fact, more significant than the value for the total worm difference. The *female* schistosomes numbered 328 from the castrated mice and 121 from the uncastrated controls. The mean adult count was 7.63 and 9.31 respectively. Although a reduction of 1.68 worms occurred, statistical analysis indicated that this difference was within the realm of chance, the "t" value being 1.54 at the 5% level.

Discussion. An important observation from the results of this investigation is that in male mice, in which the sexual hormones of the testis were altered by castration, the male schistosomes were in some way affected whereby significantly fewer adult male worms occurred than in the animals with normally functioning testis. Thus, it may be assumed that male *S. mansoni* are influenced directly or indirectly by the sex hormones in the male albino mouse.

A factor which warrants attention is that the inactivation of testosterone takes place in the liver(16-21). It is in the liver and mesenteric venules that the parasites are normally found. In this respect a direct relationship may exist whereby testosterone is necessary for normal metabolism of male schistosomes. Of course, since the liver in itself is such a vital organ with so many activities simultaneously occurring, various other factors may be considered.

One of these which may be important to the parasite is the glycogenic and glycogenolytic activity which takes place in that organ(22). Bueding(23) in significant metabolic studies of *S. mansoni in vitro* has indicated the importance of glucose intake and glycolysis to the parasite. Thus any alteration of these processes may be harmful. Another point worthy of mention is the indication that the male worms undergo a greater rate of glycolysis than do the females. They also contain more than twice as much glycogen than the females in body weight(24).

There may be many intermediate and indirect factors involved in the mechanism of the experimental phenomena presented. Further research on these problems is being undertaken, and will be reported on at a future time. It is quite feasible that new information on host-parasite relationships may be forthcoming by the study of hormonal interaction between the two.

Summary. A group of castrated male albino mice and an uncastrated control group were equally infected with *Schistosoma mansoni.* Nine weeks later the animals were sacrificed and the adult parasites collected, separated according to sex and counted. The mean adult parasite count per mouse, for each group, showed a significant reduction in the male schistosomes of the castrated animals. This difference indicates a relationship between male sex hormones and the male parasite.

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Effect of Somatotrophic Hormone and Streptomycin on Mice Exposed to Total Body X-Irradiation.* (20273)

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Selye, Salgado and Procopio(1) have reported that rats subjected to whole body x-radiation (671 r total dose in 2 exposures 6 days apart) and treated twice daily with subcutaneous injections of 1.5 mg of somatotrophic hormone (STH) gained weight, whereas untreated controls lost weight during the 2nd and 3rd weeks post-irradiation. This treatment did not, however, reduce the mortality. Since loss of weight is a well recognized effect of radiation injury(2,3), their observation suggested that treatment with STH might have exerted some beneficial effect which was not reflected in the survival rates because of the occurrence of post-irradiation infection. In fact, they noted that some of the STH treated rats at autopsy showed lesions suggestive of infection.

Infection has been shown to play a significant role in the death of mice exposed to moderate doses of x-radiation(4). It seemed possible, therefore, that any therapeutic effect of STH in the treatment of radiation injury might be more clearly demonstrable in animals protected against infection by concomitant treatment with streptomycin. Accordingly, the following experiments were made to

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* This investigation was carried out under a contract between the U. S. Atomic Energy Commission and The University of Chicago.

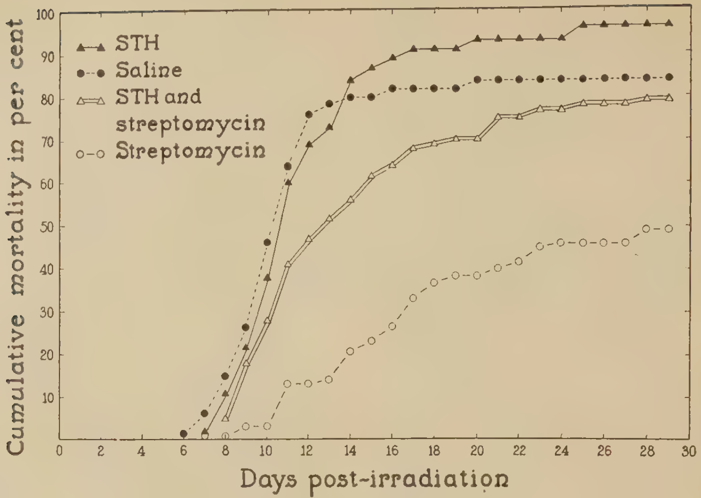

FIG. 1. Mortality curves of x-radiated mice treated with STH and streptomycin, STH alone, streptomycin alone, or saline.

explore this possibility. Irradiated mice were treated with both STH and streptomycin. Streptomycin was chosen because it had been found, by Miller, Hammond and Tompkins (5), to be the most effective of the antibiotics studied in controlling post-irradiation infection.

Methods. The mice were Rockland, female, RAP mice, 11 weeks old, weighing 20-25 g. They were housed in groups of about 20, in metal cages, the floors of which were covered with wood shavings. Rockland mouse pellets and water were available to them at all times. They were exposed to a single dose of 550 r total body x-radiation, delivered at 250 kv, 15 ma at a distance of 51.0 cms, with 0.25 mm of copper and 1.0 mm of aluminum filtration at a rate of approximately 30 r/min.[†] The mice were divided into 4 groups which were treated as follows: 81 mice were given somatotrophic hormone and streptomycin; 45 mice, somatotrophic hormone alone; 106 mice, streptomycin alone; and 45 controls, sterile saline. All injections were made subcutaneously. The first dose of 0.6 mg somatotrophic hormone[‡] was given within one hour after irradiation and repeated daily for 20 days. It was prepared fresh each day. This dose of hormone was found to produce good gains in body weight of normal mice. No evidence of toxicity appeared when this dose or more than twice this amount was given to normal mice for 5 days. Streptomycin[§] (6 mg a day) was begun on the third day post-irradiation and continued for 20 days.

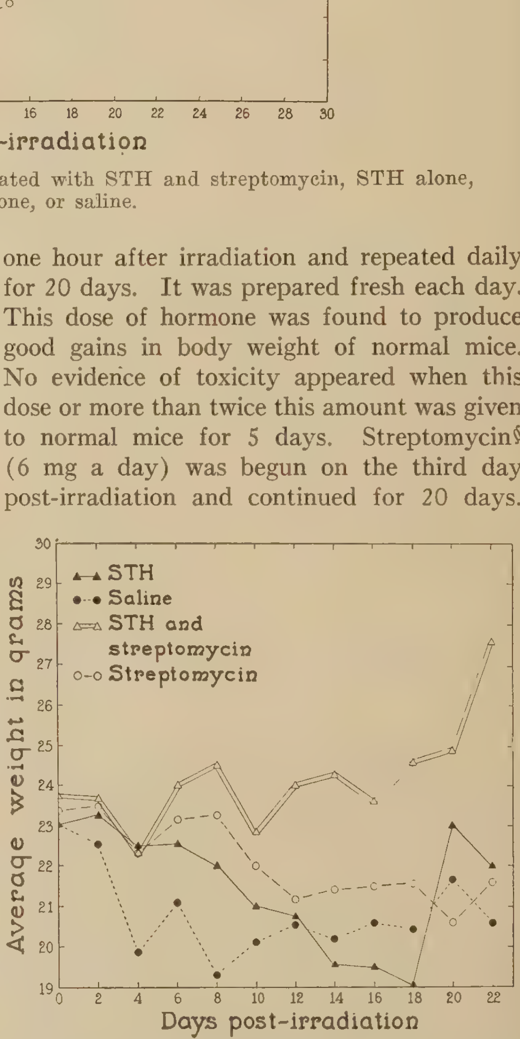

FIG. 2. Average weights of x-radiated mice treated with STH and streptomycin, STH alone, streptomycin alone, or saline.

[†] The authors are indebted to the U. S. Air Force Radiation Laboratory, University of Chicago, for use of their x-ray facilities.

[‡] Lot No. R491024 generously supplied by Dr. Irby Bunding of The Armour Laboratories, Chicago, Ill.

[§] Kindly furnished by Charles Pfizer & Co., Brooklyn, N. Y., through the courtesy of Dr. Gladys L. Hobby.

TABLE I. Incidence of Positive Blood Cultures in Autopsied Mice.

	STH and streptomycin	Streptomycin	STH	Saline
No. of mice treated	81	106	45	45
" " deaths	64	52	43	38
" autopsied and cultured	43	27	43	38
" positive blood cultures	12	5	37	38
% of blood cultures positive	28	18	86	100

TABLE II. Microorganisms Recovered from Blood Cultures of Autopsied Mice.

	Mice treated with			
	STH and streptomycin	Streptomycin	STH	Saline
Proteus	—	—	15	11
Escherichia coli	1	—	3	5
Alcaligenes fecalis	6	—	2	—
Aerobacter aerogenes	—	—	—	1
Salmonella	3	2	4	10
Enterococcus	2	3	—	3
Paracolobactrum	—	—	10	8
Beta hemolytic streptococcus	—	—	—	1

The mice were weighed in groups every other day. All mice which died were autopsied, except those which were badly mutilated when found in the morning. Heart's blood was cultured in brain-heart infusion broth (Difco) which was subcultured after 24 hours incubation onto blood agar and Eosin Methylene blue agar.

Results. Fig. 1, which plots the cumulative mortality curves, shows that the combination of STH and streptomycin did not significantly reduce the mortality (30 days) below that of the saline treated controls. The only group showing a significant reduction in mortality was that treated with streptomycin alone.

The weight curves, presented in Fig. 2, show that the saline controls lost weight most rapidly and those treated with somatotrophic hormone alone lost weight at almost the same rate. Those treated with streptomycin alone lost weight still less rapidly. Only one group —that treated with both STH and streptomycin—maintained body weight and began to gain weight by the end of the 2nd week postirradiation.

Table I shows the incidence of positive cultures of heart's blood in the four groups of mice. The smallest number of positive cultures was found in the two groups treated with streptomycin, alone or in combination with STH (18% and 28%, respectively, of the animals autopsied and cultured).

Table II lists the microorganisms recovered from the positive blood cultures. All of them, with the exception of one Beta hemolytic streptococcus, belong to the normal microflora of the lower gastrointestinal tract of this strain of mice and are essentially the same as those found by Miller, Hammond and Tompkins(4,6) to be responsible for the post-irradiation bacteremias of mice.

Discussion. These results fail to demonstrate any reduction in mortality in irradiated mice from treatment with somatotrophic hormone even when it is combined with streptomycin. In fact, the mortality among the mice treated with both STH and streptomycin was significantly higher than in those treated with streptomycin alone, even though the incidence of bacteremia was markedly reduced. It is interesting to note that the animals that had received both STH and streptomycin maintained better body weights. In spite of this finding and positive evidence of control of bacteremia, these animals died more rapidly than did those receiving streptomycin alone.

Summary. 1. Treatment with somatotrophic hormone failed to reduce the mortality of mice exposed to 550 r total body x-radiation, even when it was combined with streptomycin to control the development of post-irradiation infection. 2. The mice which received both drugs maintained body weight, but those which received either drug alone lost weight.

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Effect of Feeding Several Sulfonamides and Related Compounds on Growth of Chicks.[*] (20274)

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Stimulation of animal growth has been observed when certain non-absorbable sulfonamides, such as sulfasuxidine or sulfathalidine are fed continuously in the diet in substantial concentrations(1-6). However, the feeding of similar concentrations of absorbable sulfonamides has been found to have a deleterious or growth depressing effect(7). It was of interest, therefore, to ascertain the growth effect brought about by feeding high dietary concentrations of Gantrisin®,[†] (sulfisoxazole), a soluble, readily absorbable sulfonamide of low toxicity(8-10) and the sparingly soluble, non-absorbable γ-keto sulfones of the intestinal antiseptic type, Ro 2-0201[‡] and Ro 2-0404 (11).[§] Sulfamethazine, sulfadiazine, sulfaguanidine, sulfanilamide and sulfaquinoxaline were included for comparison. Because of its sensitivity to the neuropathological effects of sulfonamides(12) and its suitability for growth studies, the chick was chosen as the test animal.

Procedure. Day-old straight run sexed single comb White Leghorn chicks[||] were placed on raised wire floors in electrically heated brooders and were maintained on the basal ration for 3 days. The basal ration used was an antibiotic-free[¶] commercial chick starting mash. After 3 days acclimatization on the basic diet, all runts and giants were discarded, the birds were wing banded and grouped into equally weighted groups of 20, containing equal numbers of males and females. The groups of birds were then placed on the experimental rations and maintained so in the brooders to the age of 4 weeks, after which time they were transferred to growing cages where they remained until the termination of the experiment. All birds were weighed weekly. Water and feed were provided *ad libitum.* The lights were controlled to keep the room well illuminated between the hours of 5 a.m.

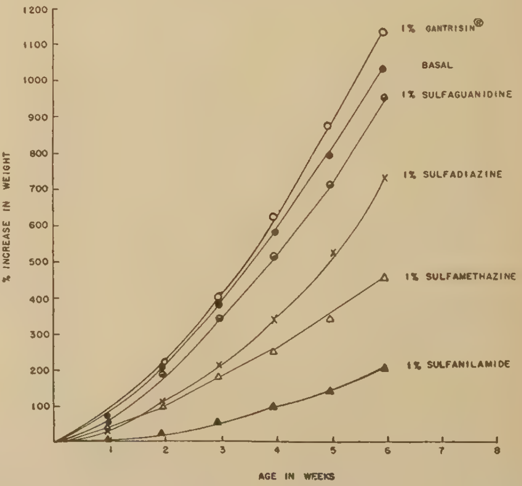

FIG. 1. Rate of increase in growth of chicks fed diets containing 1% sulfonamide.

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[*] Roche Publication No. 344.

[†] 3,4-Dimethyl-5-sulfanilamido isoxazole.

[‡] 4-Phenyl-4-sulfanilyl-2-butanone.

[§] β-Phenyl-β-sulfanilyl-propiophenone.

[||] Obtained from Kerr Chickeries, Frenchtown, N. J.

[¶] "Start and Grow" chick mash, manufactured by GLF Feed Co-Op Mills.

TABLE I. Relative Weight Responses of White Leghorn Chicks at 6 Weeks when Fed Sulfonamides Continuously in the Diet.

Compound	% conc. in diet	Avg wt ± S.E.*	% deviation from control†	Exp. No.
Sulfisoxazole‡	1	460 ± 9	+12	8
	.3	437 ± 6	+ 6	8
	.1	477 ± 10	+12	7
	.1	448 ± 6	+ 9	8
	.1	543 ± 9	+ 6	9
	.03	447 ± 5	+ 5	7
	.03	551 ± 5	+ 7	9
	.01	465 ± 12	+ 9	7
	.01	520 ± 11	+ 1	9
Sulfaquinoxaline	.3	206 ± 11	—60	9
	.1	421 ± 8	—18	9
	.05	387 ± 8	— 6	8
	.025	391 ± 10	— 5	8
	.0125	493 ± 8	— 4	9
Sulfanilamide	1	108 ± 10	—79	9
	.3	350 ± 12	—32	9
	.1	423	— 1	7
	.03	427	0	7
	.01	435	+ 2	7
Sulfamethazine	1	181 ± 16	—56	8
	.03	325 ± 9	—21	8
	.1	493 ± 9	— 4	9
	.03	473 ± 8	— 8	9
	.0125	509 ± 10	— 1	9
Sulfadiazine	1	350 ± 9	—32	9
	.3	463 ± 14	—10	9
	.1	524	+ 2	7
	.03	499	— 3	7
Sulfaguanidine	1	371 ± 11	—10	8
	.3	404 ± 9	— 2	8

* Stand. error of mean: $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

† Avg wt of control groups

	g	S.E.*
Exp. 7	427	±10
8	412	± 9
9	514	±12

‡ Gantrisin ®

and 9 p.m. Humidity and temperature varied with the weather and the temperature ranged from 22 to 28°C. The experimental diets were made by mixing thoroughly the desired amount of a compound into the basal diet. All compounds were employed as the commercially available free form of the drug.

Results. The rates of increase in weight of birds fed diets containing 1% of the sulfonamide are shown in Fig. 1. It is interesting to observe from these data that Gantrisin® effects a slight enhancement in growth over the control whereas sulfanilamide, sulfamethazine, sulfadiazine and sulfaguanidine depress growth. After 6 weeks of continuous feeding of 1% of the sulfonamide in the diet, the birds getting Gantrisin® gained 12% in weight over the drug free control, whereas birds getting sulfanilamide showed a growth repression of 79% and those getting sulfamethazine showed a growth repression of 56%. Birds fed 1% sulfadiazine and 1% sulfaguanidine showed growth repressions of 32% and 10% respectively. With diets containing 0.3% of drug, Gantrisin® still exerts a small growth enhancing effect. The growth depressing effects of sulfaguanidine and sulfadiazine are considerably reduced whereas sulfanilamide and sulfamethazine are still markedly inhibitory to growth. Of the compounds examined in this series, sulfaquinoxaline was the most inhibitory to growth.

TABLE II. Growth Response Obtained on Adding Ro 2-0201* or Ro 2-0404† to Basal Ration.

Compound	% conc. in diet	Total wt at 5 wk ± S.E.	% gain over control
	Basal	436 ± 9	—
Ro 2-0404	.03	471 ± 8	8
	.1	500 ± 7	15
Ro 2-0201	.03	498 ± 7	14
	.1	488 ± 8	12
	.3	489 ± 8	12

* 4-phenyl-4-sulfanilyl-2-butanone.

† β-phenyl-β-sulfanilyl-propiophenone.

It was of interest to ascertain if any of the compounds tested might be stimulatory to growth at markedly reduced dietary concentrations. It may be seen from the data in Table I that a stimulation of growth was not observed when sulfamethazine, sulfadiazine or sulfaquinoxaline were fed at dietary levels as low as .01% and sulfadiazine as low as .03%.

Statistically significant growth stimulation was obtained when small amounts of Ro 2-0404 and Ro 2-0201 were added to the basal ration. These results may be seen in Table II. It is of interest to note that Ro 2-0201 exerts a maximum stimulatory effect at a diet level of .03% and maintains this effect at levels of up to 10 times this concentration. Diet concentrations lower than .03% were not tested.

Discussion. In our experiments, no evidence of drug toxicity was observed when Gantrisin® (sulfisoxazole) was fed continuously for 6 weeks at high diet concentration to young chicks. In fact, Gantrisin® was observed to produce a stimulation of growth when added to the diet in concentrations ranging from 0.03% to 1%. These findings stand in marked contrast to the growth depressing effects observed when sulfamethazine, sulfadiazine, sulfanilamide, sulfaguanidine, or sulfaquinoxaline were fed to chicks at comparable or even markedly reduced levels, and no growth stimulation was observed to occur with any of the above compounds at any level tested.

The toxicity of sulfamethazine for the chick has also been shown by Asplin *et al.*(13), Levine and Barber(14), and Waletzky and Hughes(15). The toxic effects resulting from the continuous administration of sulfadiazine, sulfanilamide or sulfaguanidine are well known(7,16,17). Our findings are in agreement with the observations(18) that sulfaquinoxaline is relatively non-toxic when fed continuously in the diet at a concentration of 0.03% but higher concentrations may repress growth.

The growth-stimulating activity of low diet concentrations of Ro 2-0201 and Ro 2-0404 is of considerable interest since these compounds are quite insoluble and are only very slightly, if at all, absorbed from the intestinal tract. It has been shown(19) that in spite of their low solubility, these compounds exert a definite anti-coli effect on the intestinal microbial population of the mouse. From the data obtained in our experiments it would seem that the presence of these compounds in the diet has favored the development of an intestinal flora highly favorable to the chick.

Summary. 1. Young chicks fed diets containing Gantrisin® (sulfisoxazole) at levels of up to 1% of the diet showed no physical abnormalities, were more uniform and thrifty in weight and appearance than the controls and weighed heavier than the controls at 6 weeks of age. 2. Young chicks fed diets containing .03% of Ro 2-0404 or from .03 to 0.3% of Ro2-0201 were also thriftier in appearance and heavier than the controls and showed no physical abnormalities. Young chicks fed diets containing 1% or 0.3% of sulfamethazine, sulfadiazine, sulfanilamide, sulfaguanidine or sulfaquinoxaline were markedly retarded in their growth.

The authors wish to express their appreciation to Dr. J. A. Aeschlimann and Dr. J. C. Bauernfeind for numerous helpful suggestions and to Dr. M. W. Goldberg and Dr. A. Rachlin for supplying the Ro 2-0201 and Ro 2-0404.

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Non-Protein Nitrogen Changes in Serum and Plasma of Rats Following Thermal Injury.* (20275)

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Disturbances in nitrogen metabolism are commonly observed after severe burns. Azotemia is one of the principal features of the metabolic changes. In general, the distribution of the elevated blood non-protein nitrogen is normal, but, occasionally, in patients(1,2) and rats(3,4) with very extensive deep burns, a large part (as much as 80%) of the azotemia is due to abnormal amounts of "residual" nitrogen whose nature has not been determined. Duval *et al.*(5) and Lambret *et al.*(6) postulated that severe burns result in a polypeptidemia. They based their conclusions on an observed increase of that amino nitrogen which is soluble in trichloracetic acid, but precipitated by phosphotungstic acid. Christensen(7) has presented evidence which casts doubt on the reliability of such determinations and interpretations.

In the present investigation, ultrafiltrates of plasma and serum from normal and thermally injured rats were utilized in a study of the non-protein nitrogen changes. In addition to the routine chemical measurements, absorption spectra in the ultraviolet were obtained, and changes of individual amino compounds were followed by ion exchange chromatography.

Methods. A. *Thermal technic.* Male albino rats of the Wistar strain, weighing between 225 and 275 g were anesthetized with ether and thermally injured deeply over about 30% of the body surface by dipping their backs in hot water (90°C) for 35 seconds(8). Rosenthal and McCarthy(4) have found that the increase in plasma residual nitrogen in similarly injured rats is at a maximum 10 to 12 hours after trauma. Accordingly, in the present study 11 to 12 hours after injury, the surviving rats (80% of those injured) were sacrificed, and their blood collected by heart punctures. Control rats, anesthetized but not burned, were studied simultaneously. Purina chow and water were offered *ad libitum* to all rats. B. *Chemical and spectrophotometric measurements.* Bloods from 16 injured rats and from 10 control rats were separately pooled and allowed to clot in vessels immersed in ice water. The bloods were then spun in a refrigerated centrifuge, and the sera filtered at 3°C through "Visking" sausage casing under 40 lb per square inch of nitrogen in an ultrafiltration apparatus made of Monel. All subsequent manipulations were performed within 24 hours. Serum ultrafiltrates were used since anticoagulants and protein precipitants generally absorb light in the region 220-290 mμ, seriously interfering with spectrophotometric measurements carried out in this wavelength range. The ultrafiltrates were analyzed for total filterable nitrogen, urea nitrogen, and amino nitrogen before and after acid hydrolysis with 6 N HCl. The hydrolyses were carried out in sealed tubes heated at 110° for 12 hours. The analyses were performed by the methods of Sobel *et al.*(9,10). Since many naturally occurring nitrogenous substances show characteristic spectral changes when irradiated with ultraviolet light, absorption spectra of the ultrafiltrates of the sera from normal and injured rats were obtained before and after ultraviolet irradiation in sealed silica cells. An Argon-Mercury lamp, 80% of whose output was at 254 mμ, was used. The absorption spectra of the ultrafiltrates were obtained with a Beckman DU spectrophotometer. C. *Chromatographic measurements.* The bloods from 20 injured rats and 20 control rats were separately pooled in vessels immersed in ice water. Sodium citrate (2.5%) in a 1:5 dilution with the blood was used as an anticoagulant. The bloods were centrifuged within a few minutes after collections. The plasma was separated from the cells and immediately frozen in 5 ml vials. For analysis, the frozen plasma was thawed at 3°C, and ultrafiltration immediately carried out. Changes in individual amino compounds were followed by an ion exchange chromatographic technic employing Dowex-50 and a colorimetric ninhydrin method, both developed by Stein and Moore(11,12). All hydrolyses were carried out in sealed vials with 6 N HCl at 110°C for 20 hours. The resultant hydrolysates were evaporated by heating under a stream of air. The dry residue was then made up to about 3 ml with H_2O, and the pH adjusted to approximately 2.5 with 2 N NaOH.

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* The experimental work was done at the Medical College of Virginia.

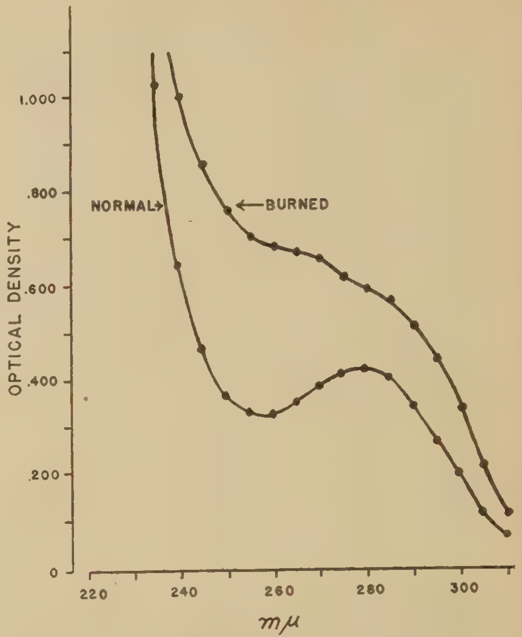

FIG. 1. Absorption spectra of sera ultrafiltrates of normal and thermally injured rats.

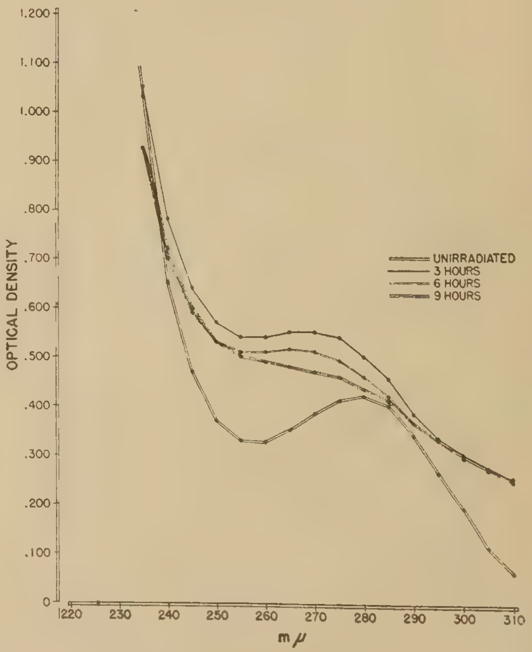

FIG. 2. Effect of ultraviolet irradiation on absorption spectrum of serum ultrafiltrate of normal rats.

Results. I. *Spectrophotometric measure-*

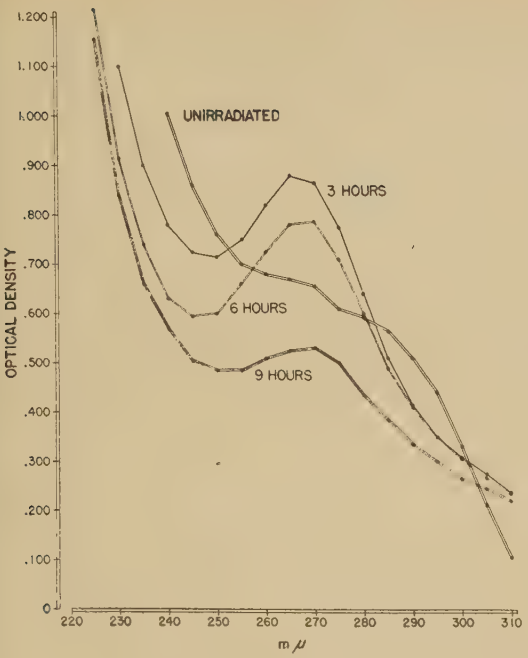

FIG. 3. Effect of ultraviolet irradiation on absorption spectrum of serum ultrafiltrate of thermally injured rats.

ments. The absorption spectra of the sera ultrafiltrates are shown in Fig. 1. The ultrafiltrate of the sera from burned rats shows a markedly altered spectrum. There is an increase in absorption over the entire range of wavelength, but especially in the region of the normal minimum at 255 mμ. The changes in the spectrum of the filtrate of normal rat sera after 3, 6, and 9 hours of irradiation are shown in Fig. 2. There is an initial rise of the entire spectrum, particularly in the region of minimum absorption (255 mμ). With further irradiation, there occurs a slight drop in the spectral pattern. When the serum ultrafiltrate of injured rats is irradiated, there is initially a decrease in the region 240-250 mμ, and a marked increase in the region 255-275 mμ (Fig. 3). With further irradiation, there is a uniform fall, so that after 9 hours of irradiation, the entire spectral pattern is lower than that of the unirradiated sera ultrafiltrate. II. *Chemical measurements.* The results of the chemical measurements are shown in Table I. There are concomitant rises in concentrations of plasma urea, and free and combined amino nitrogen. III. *Chromatographic measurements.* A chromatogram of normal pooled rat plasma ultrafiltrate is shown in Fig. 4. The separations are, in general, clear cut. The following features of the chromatographic patterns should be noted: 1) The first component, or forepeak stable to acid hydrolysis, was found in all plasma ultrafiltrate specimens. It has been tentatively identified as taurine. 2) The second and sixth peaks (Fig. 4) are unstable to acid hydrolysis. The first of these peaks is an amino conjugate. Its composition will be discussed later. The sixth peak was studied by isolating it from single runs of ultrafiltrate, hydrolyzing, and rechromatographing (Fig. 5). It is composed of asparagine, serine, and glutamine. 3) There was no measurable cystine in any specimen. 4) In some runs, the histidine peak showed resolution into two components. It is probable, therefore, that the "histidine" figures are high, since they may include another component. 5) Tryptophan is destroyed by the acid buffers employed. 6) The basic substance immediately preceding lysine was found in all samples. It does not yield the typical amino-ninhydrin purple color, but rather a blue-green one. Its identity has not been established. 7) Arginine could not be separated from other basic, ninhydrin reactive material. Chromatograms were therefore stopped after lysine.

Table II shows the similarity of the results of the chromatographic separations and colorimetric ninhydrin determinations of some amino acids of normal rat plasma as compared with measurements made by a microbiological method(14). The results obtained by

TABLE I. Effect of Severe Thermal Injury on Nitrogen Partition of Rat Serum Ultrafiltrate. (mg N/100 ml of serum ultrafiltrate.)

	Total filterable N	Urea N	Amino N	Amino N after hydrolysis	Combined amino N	Combined amino N as % of total filterable N
Normal	28.3	14.0	6.3	11.8	5.5	19
Burned	130	76.0	18.9	47.3	28.4	22

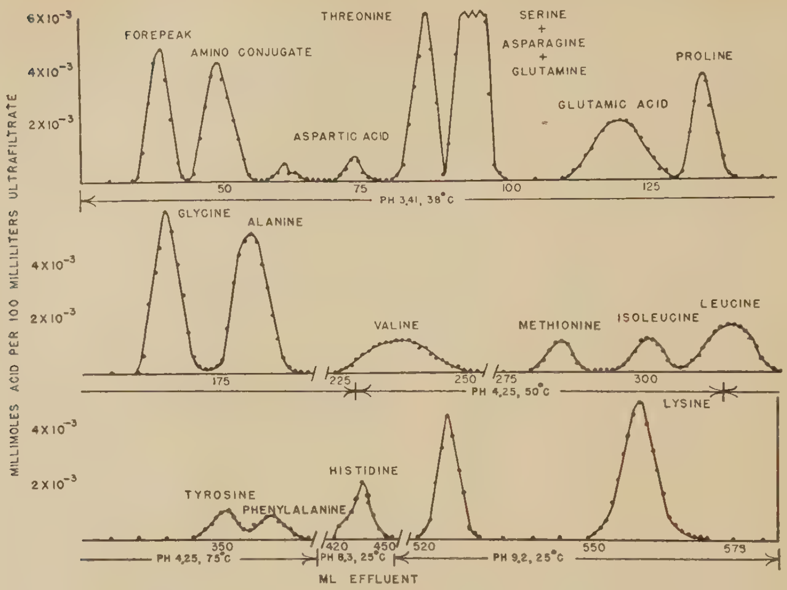

FIG. 4. Chromatographic pattern of normal rat plasma ultrafiltrate.

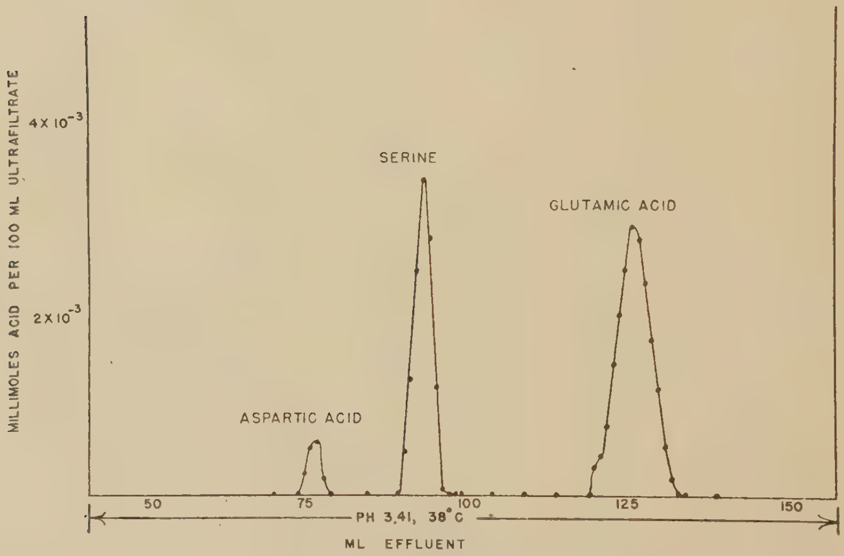

FIG. 5. Pattern of hydrolyzed "serine + glutamine + asparagine" peak isolated from normal rat plasma ultrafiltrate.

chromatographing plasma ultrafiltrates of injured rats are also listed in Table II. In general, the amino acid concentrations are elevated in the injured animals. The most striking effects are the increases of the forepeak and the amino acid conjugate. These two components account for 80% of the increase of amino nitrogen in rat plasma after burning.

About 20 ml of normal plasma were used for isolation of the amino conjugate. Hydrolysis of this fraction and rechromatographing yielded the pattern shown in Fig. 6. The predominant components are an acid stable fraction (as yet unidentified), glycine, and glutamic acid, and lesser amounts of alanine, proline, serine, threonine, valine, and leucine. The distribution of amino acids of the con-

TABLE II. Concentration of Amino Compounds in Normal and Thermally Injured Rat Plasma Ultrafiltrates. (mM amino acid/100 ml plasma ultrafiltrate.)

	$\times 10^{-3}$			
	Normal			
	Chromatographic method	Microbiological method, Henderson *et al.*(14)*	Burned	% change
Forepeak (Taurine?)	39.0		149.8	+284
Amino conjugate	40.0†		127.6	+219
Aspartic acid	4.5		4.3	— 4
Threonine	37.7	37.6	31.7	— 16
Serine	21.8		25.9	+ 18
Glutamine	27.5		28.3	+ 4
Asparagine	3.7		5.2	+ 40
Glutamic acid	22.1		18.9	— 14
Proline	28.5	37.1	17.7	— 40
Glycine	52.7		45.4	— 15
Alanine	57.1		64.5	+ 12
Valine	24.9	22.8	20.1	— 20
Methionine	6.7	6.4	6.3	— 6
Isoleucine	10.3	10.0	15.1	+ 50
Leucine	17.3	20.3	25.6	+ 48
Tyrosine	8.7	12.3	17.6	+113
Phenylalanine	8.4	8.3	18.6	+125
Histidine	13.0‡	6.3	37.0	+185
Lysine	37.2	39.7	38.0	+ 2
Totals	461.1		697.6	

* These data appear as μg amino acid/ml plasma in Henderson *et al.*(14).
† Figures include *only* free amino nitrogen, calculated as leucine.
‡ Results probably high. See text.

jugate isolated from injured rats' plasma is similar to the normal. It is possible that the isolation and subsequent analysis of much larger amounts of the conjugate would reveal small amounts of other amino acids.

Discussion. The absorption spectrum of the ultrafiltrate of the sera from normal rats is typical of a mixture of amino acids, free or combined. The rise between 265-290 mμ is probably due in large part to amino acids containing the benzene nucleus(13). The absorption peaks of phenylalanine, tyrosine, and tryptophane are 260 mμ, 280 mμ, and 280 mμ, respectively. The ultrafiltrate of the sera from injured rats shows a markedly altered spectrum. There is an increase in absorption over the entire range of wavelength, but especially in the region of the original minimum at 255 mμ. This is consistent with a change, qualitative and/or quantitative in the sera amino acids free and combined, and with the appearance of purine containing compounds. At a pH of about 7 the absorption peaks of guanine, adenine, and xanthine are about 260 mμ while the main peak of uric acid is 293 mμ.

The changes observed in the spectral patterns after ultraviolet irradiations of the sera ultrafiltrates of the normal and injured rats are also compatible with the idea that there are changes in the purines and amino acids after injury. It has been shown that when proteins are irradiated with ultraviolet light the extinction coefficient increases over the whole range of wavelengths in the ultraviolet region, but especially in the region of the original minimum, around 250 mμ(15). McLean and Giese(15) have shown these effects can be attributed to modifications of the aromatic amino acids. When purines are irradiated with ultraviolet light, there is a progressive diminution of the absorptive patterns(16).

Proportionate concentration changes of the purines and aromatic amino acids cannot be inferred from the absorption curves (Fig. 1) since the extinction coefficients of these compounds are different, the purines, in general, having the higher absorption. Green and his associates(17) have found that various forms of trauma, including thermal injury, are accompanied by an increase of nucleotide metabolites in the plasma. This rise is quantita-

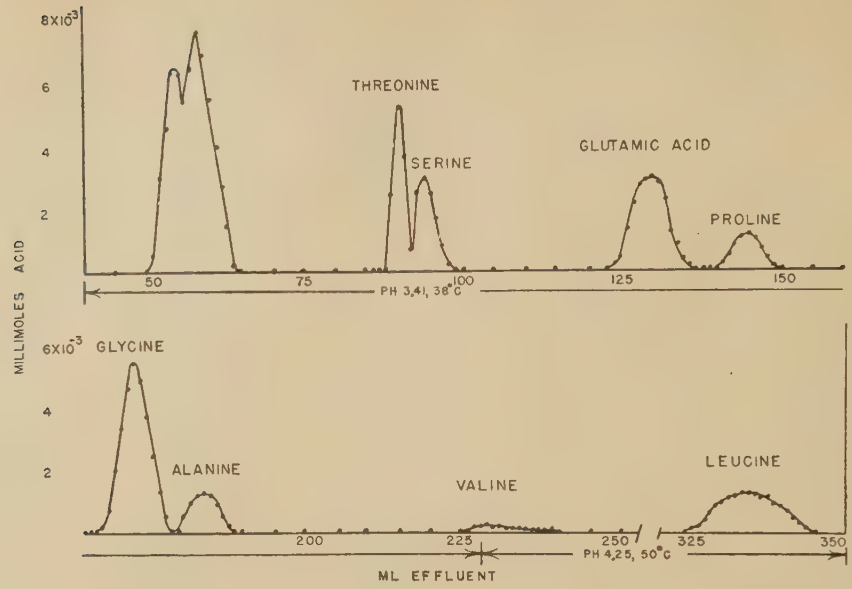

FIG. 6. Pattern of hydrolyzed amino conjugate isolated from normal rat plasma ultrafiltrate.

tively small in relation to that of amino nitrogen compounds. We have observed that almost the entire increase of filterable nitrogen in the sera of rats after burning consists of urea and free and combined amino nitrogen (Table I) in their normal relationships. As pointed out by Christensen(7) part of the increase in combined amino nitrogen might be due to heightened proteolytic activity and subsequent formation of peptides from the serum clot. An attempt was made to minimize this action by keeping the blood cold at all times, and centrifuging in the cold immediately after the blood was collected. Further, the value for hydrolyzable nitrogen (28.4 mg %) in the ultrafiltrate of serum from injured rats is almost identical with the "residual" nitrogen (30 mg %) found in plasma of rats given an identical thermal injury (Rosenthal and McCarthy(4)). This correspondence between plasma and serum values indicates that enzymatic formation of peptides from fibrin *in vitro* is not a significant factor in explaining the findings.

There is a close correspondence between our results for the amino acids of plasma ultrafiltrates of normal rats and those obtained by Henderson *et al.*(14) for rat plasma amino acids using microbiologic technics (Table II). Identical results are not to be expected, since a number of conditions were different in the two studies: 1) The microbiological measurements can be expected to include some peptide nitrogen. This would tend to raise the results for some acids, as compared with the chromatographic method, which, in this respect, is more specific. 2) The method by which protein is separated from the plasma is of fundamental importance in these determinations. Henderson *et al.*, who used the tungstic acid precipitation method, found that for given samples of plasma, the amino acid content increased, on the average, about 200% after acid hydrolysis. Our plasma ultrafiltrates yielded only an additional 50% of amino nitrogen after hydrolysis. It seems clear that our filtrates contained less combined amino nitrogen. 3) Our figures are reported as concentration of amino acids per unit of ultrafiltrate, while the above workers reported their results as concentration per unit of plasma. Our figures in this respect, then, will be higher than theirs.

Asparagine, isoleucine, leucine, histidine, tyrosine, and phenylalanine are increased in the sera ultrafiltrate of injured rats. The increases in the two aromatic amino acids are consistent with the spectrophotometric observations. Quantitatively, the largest part of the increase in amino nitrogen is made up of the forepeak (taurine?) and the amino acid conjugate. Some of the amino acids, for example proline and valine, are decreased in the injured animals.

The composition of the amino acid conjugate is striking, since it includes only a few of the common acids. Also, there is a large residual fraction unaffected by the hydrolysis procedure. The distribution of amino acids of the conjugate is similar in normal and injured rats. A peptide was isolated from a number of tissues of various animals, including the rat, by Borsook *et al.*(18). This peptide contains most of the common amino acids. It seems likely that the peptide fraction is a heterogeneous one, including large and small molecules. There is probably no sharp dividing line between "protein" and "non-protein" nitrogen in rat plasma. The efficiency with which the plasma is "deproteinized" is of fundamental importance in the interpretation of the results of "peptide" analyses. The heat coagulation procedure used by Borsook and his associates might account for the relatively large "peptide" residuum which they report.

Summary. 1. Ultrafiltrates of plasma and serum from normal and thermally injured rats were utilized in a study of non-protein nitrogen changes following thermal injury. The rats were burned deeply over about 30% of the body surface and the bloods were collected 11-12 hours after injury. 2. Absorption spectra were obtained before and after *in vitro* ultraviolet irradiation of the sera ultrafiltrates. The changes observed in the spectrum of the sera ultrafiltrate of the injured rats is consistent with a modification qualitative and/or quantitative in the sera amino acids, free and combined, and with the appearance of purine containing compounds. 3. Almost the entire increase in filterable nitrogen in the sera of rats after thermal injury consists of urea and free and combined amino nitrogen in their normal relationships. 4. Measurements of specific amino compounds were carried out by ion exchange chromatographic and colorimetric ninhydrin technics. The results obtained by these methods for the amino acids of plasma ultrafiltrates of normal rats are similar to values obtained by others for rat plasma amino acids using microbiologic technics. 5. In the plasma ultrafiltrate of injured rats, there are increases of some amino acids, particularly tyrosine, phenylalanine, and histidine, while other amino acids are decreased. Quantitatively, the largest part of the increase in amino nitrogen is made up of a forepeak (taurine?) and an amino acid conjugate. 6. The amino acid conjugate includes a large fraction unaffected by hydrolysis and a few of the common amino acids. The composition of the conjugate is similar in normal and injured rats.

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Effect of Semi-Starvation on Propulsive Motility of the Small Intestine. (20276)

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A number of workers have reported the effects of fasting or of semi-starvation on gastric motility. There are but few reports in the literature, however, on the effect of the above states on the propulsive motility of the small intestine.

Our object was to study the effect on the propulsive motility of the small intestine of rats which had been placed on a semi-starvation diet and had lost a pronounced degree of weight. Such a study is of academic and clinical interest since chronic illnesses may cause undernutrition and certain peoples chronically experience semi-starvation.

Methods. Twenty-four adult male albino rats (Wistar-strain) weighing 196-262 g (avg. 217 g) were used—12 as controls and 12 subjected to semi-starvation. Each rat was housed in a separate cage and kept in a 72°F environment. An adequate diet and water were given *ad libitum* to the control animals. The experimental group was allowed water *ad libitum*, but received progressively less of the same diet for an average of 19 days (16-22 days) so weight was progressively lost. When they had lost the desired amount of body weight and showed definite signs of physical weakness they were starved for 12 hours and the propulsive motility of the small intestine studied. Each semi-starved rat was paired with a control animal, which had been starved for the same length of time. Each member was given 2 cc of a mixture of 10% powdered charcoal suspension in 10% gum acacia solution by stomach tube. Forty minutes later the animal was killed, the small intestine removed and the distance the charcoal-mixture had traversed the gut measured.

Results. The experimental rats weighed an average of 225 g at the start, semi-starvation reduced this to 137 g, an average loss of 39%. During this period the controls gained from 208 g average weight to 262 g, a 26% gain. Motility was as follows: The charcoal traversed an average of 72% of the gut in the controls, and an average of 91% in the semi-starved. The difference is significant at the 0.1% level (t test).

Discussion. Menville(1) found intestinal motility in rats stimulated by semi-starvation (using a buttermilk barium sulfate mixture). Our results are in agreement. Jonas(2) in man and Dagaew(3) in dogs found gastric motility influencing intestinal motility. Other evidence(4) indicates that the stomach of dogs may be severely depressed by hypoxia without affecting intestinal motility. The mechanism of intestinal stimulation by semi-starvation is therefore obscure; there is a possibility that the hypoglycemia known to occur(5) may directly affect the intestine.

Summary. The effect of semi-starvation on the propulsive motility of the small intestine of albino rats was studied by Macht's technic. At the end of 40 minutes, the charcoal-mixture traversed 72% of the small intestine of the control animals, and 91% in the semi-starved animals.

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Characteristics of a Psittacosis Viral Agent Isolated from a Turkey.* (20277)

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In an attempt to find the source of the recent (1952) outbreaks of clinical psittacosis among employees of poultry-dressing plants (63 cases with 4 deaths), the sera of 126 turkeys from 7 farms were tested by the complement fixation inhibition method (Karrer *et al.*(1)); 25% were positive reactors. Attempts to isolate a viral agent were at first unsuccessful, but when suspensions prepared from the lung and kidney tissues of one turkey (No. 05404) were inoculated subcutaneously and intranasally into mice, a viral agent morphologically indistinguishable from the classical strains of the psittacosis group was isolated. A comparative study conducted according to a standard routine procedure (Meyer and Eddie(2)) identified the strain as a characteristic member of this group.

Morphology. The characteristics of the viral agent isolated from a turkey are as follows: Morphologically the impression preparations made from the peritoneal exudate, from the lung of infected mice or from the yolk sac of inoculated embryonated eggs showed Castaneda-positive elementary bodies, smaller than those formed by other viruses of this group, with the usual developmental stages in the cytoplasm, readily rendered visible by the Macchiavello stain.

Pathogenicity. The infection spectrum of the agent is broad and the pathogenicity is high. *Mice* infected by the intraperitoneal route with a virus dilution of 10^{-8} and occasionally 10^{-9} died in from 4 to 5 days; dilutions of 10^{-4} and 10^{-5} given intranasally were regularly lethal in 6 days; after intracerebral inoculation death occurred on the 5th to 6th day with dilutions of 10^{-7}; on subcutaneous injection with a dilution of 10^{-1} occasionally a few mice succumbed to infection between the 6th and 15th day. All mice that recovered from infection were carriers. Intravenous injection killed in from 6 hours to 15 days, depending on the dilution of the tissue suspensions. Yolk sac viral dilutions of 10^{-8} sometimes killed in 4 to 15 days. *Guinea pigs* receiving by the intraperitoneal route 1 ml of a 20% suspension of infected tissues (liver and spleen or yolk sac) succumbed regularly in 3 to 8 days. *Parakeets* and *ricebirds* on intramuscular injection with a low viral dilution (10^{-1}) presented at autopsy on the 5th or 6th day typical lesions of psittacosis. Three of six 7-day-old *chick embryos* infected by the yolk sac route with 10^{-8} dilution succumbed on the 3rd day, further attesting to the high virulence of this agent. Infected yolk sacs harvested shortly before death, after draining off excess yolk and diluting in phosphate buffered gelatin solution, were highly toxic on intravenous inoculation of mice weighing 10 to 12 g. In dilutions of as high as 1:800, 100% of the animals succumbed, after having had symptoms of lethargy and labored breathing, within 5 days. The ears, paws and tails of those surviving 72 hours were often deep red. The striking capillary dilations in the skin and in part also in the viscera are associated with hemolysis of red cells and are usually observed when highly toxic psittacosis agents are tested by the intravenous route. In rare instances fading of the redness of the ears heralded recovery, but the mice were found to be carriers of the agent when sacrificed several weeks after the infecting injection. The *toxin* of the turkey strain shares all characteristics of the toxins of the psittacosis-lymphogranuloma venereum agents. The death rate following injection of the toxin of the turkey strain is shown in Fig. 1 and 2.

Antigenic relationships. Antisera from infected psittacine birds, pigeons, sheep or man have given complement fixation reactions when the turkey strain was used as antigen. Sera from turkeys or roosters infected with the turkey strain bound complement in the

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* Through the courtesy of Dr. I. V. Irons, Texas State Department of Health, Austin, this viral agent was made available to the Hooper Foundation for study.

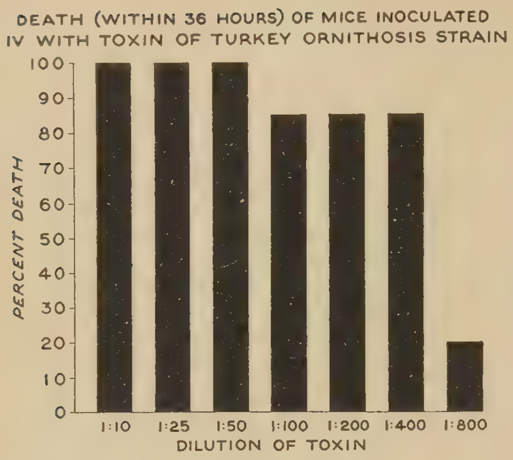

FIG. 1.

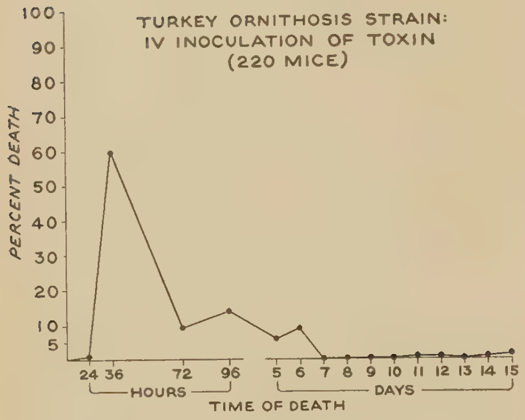

FIG. 2.

presence of Cocto-antigens prepared from avian or mammalian strains of the psittacosis group. Thus the strain gives reciprocal serologic cross reactions.

Guinea pigs surviving infections with an avian strain (egret SE45), as evidenced by complement fixation tests, were completely resistant to intraperitoneal challenge with the turkey strain, which induced death in control animals. On the other hand, depending on the host origin of the mammalian strains employed to immunize guinea pigs, the resistance to re-infection with an avian strain varied from 20 to 60%.

Hyperimmunization of roosters with toxoids prepared from highly toxic yolk sac suspensions (40 ml of a 20% suspension in 25 injections over a period of 2 months) when bled on the 15th day after the last injection (75th day) yielded antisera which reacted in the complement fixation inhibition test in dilutions of 1:32 and even 1:128 (rooster no. 19). Such antisera diluted 1:10 and mixed with 2 LD_{50} doses of toxin in the *in vitro* neutralization test (Rake and Jones(3)) protected all mice against the turkey toxin for at least 48 hours. As Table I shows, the turkey antiserum also neutralized to a less pronounced degree toxins prepared from the human Louisiana (Borg) and egret (SE45) strains. It exerted no protection against the toxins derived from 2 pigeon and 1 bovine encephalomyelitis virus. On the 6th day after injection of the mixture of antiserum and viral agent, when the anti-infectious properties of the turkey serum became active, the apparent relationship of the Louisiana and egret strain had disappeared. In one test 40% of the mice inoculated with turkey serum dilutions and 2 LD_{50} doses of toxin containing 100 million infectious units survived not only intoxication but also infection. At autopsy on the 20th to 30th day the mice were free from latent carrier stages. Thus the *in vitro* contact of the turkey virus with its homologous antiserum proved that not only the toxic, but equally the infectious properties were specifically neutralized. The specific protective action of an anti-psittacosis serum is more definitely demonstrated by intravenous injection than by intranasal instillation or intracerebral or intraperitoneal injection of the mixture (Hilleman(4)). Depending entirely on the potency of the antiserum, even 400 million infectious particles were rendered non-infectious when brought in contact with the antibodies.

To check these results, the turkey toxin was mixed *in vitro* with antisera against other members of the psittacosis-lymphogranuloma venereum group. The data in Table II show that the turkey antiserum again neutralized both the toxin and the infectious elementary bodies and protected 50% of mice from death after the 6th day. An anti-Louisiana or anti-egret strain serum neutralized turkey toxin for up to 36 hours (30 to 45% of the animals survived) but these antisera, except in high concentrations, could not render the infectious particles inactive. The serum prepared with a parakeet strain (N.Y.) or with a cat

TABLE I. Neutralization of Toxin of Turkey Strain by Specific Antisera. Survival rate of mice inoculated intravenously.*

Strains used to prepare antiserum	Total mice	Rate†										
		Hr				Days						
		24	36	72	96	5	6	7	8	9	10	21‡
Turkey	20	100	90	70	65	55	50	40	40	40	40	40
Egret (SE45)	40	100	60	45	30	15	10	0				
Human (La., Borg)	40	100	72	37.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Parakeet (N. Y.)	40	95	45	22	15	7.5	5	5	5	5	5	5
Duck (Burns)	40	95	55	30	20	15	7.5	7.5	7.5	5	0	
Cat (PP)	40	90.5	40	25	7.5	5	2.5	2.5	2.5	0		
Bovine (BEV)	40	45	0									
Controls	40	70	22.5	0								

* Antiserum diluted 1:10, toxin in 2 LD_{50} doses.

† Calculated on percentage distribution.

‡ Mice surviving until 21st day sacrificed and examined.

TABLE II. Neutralization of Specific Toxins by Antiserum against the Turkey Strain. Survival rate of mice inoculated intravenously.*

Strains used to prepare toxins	Total mice	Rate†										
		Hr				Days						
		24	36	72	96	5	6	7	8	9	10	21‡
Turkey	20	100	90	70	65	55	50	40	40	40	40	40
Egret (SE45)	40	90	30	20	15	15	0					
Human (La., Borg)	40	90	45	10	5	0						
Pigeon (P207)	40	25	0									
Pigeon (Au46)	40	50	0									

* Turkey strain antiserum diluted 1:10, toxin in 2 LD_{50} doses.

† Calculated on percentage distribution.

‡ Mice surviving until 21st day sacrificed and examined.

strain (PP) were less active with respect to toxin and infection neutralization than those of the highly virulent Louisiana strain. Two pigeon and 1 bovine encephalitis antisera did not neutralize the turkey toxin.

Conclusions. It has been demonstrated that the psittacosis (ornithosis) viral agent isolated from a turkey is antigenically specific. The Louisiana human, the egret and the turkey strain have in common an endotoxic component probably responsible for their high virulence for man and for their broad infection spectrum. Epidemiologic experience has proven such strains to be the cause of serious outbreaks of pneumonitis in inhabitants of the Louisiana bayou country (Olson and Larson (5)), in poultry dressers and in laboratory workers. Until other strains isolated from turkeys have been carefully compared with other psittacosis viral agents from southeastern United States it is premature to conclude that turkeys are the sole hosts of ornithosis agents of virulence greater than that of strains found in pigeons, ducks, chickens and even psittacine birds. Finally, there is no urgency to burden the unfortunate classification of the psittacosis-lymphogranuloma venereum group with a new species when the technic of neutralization of viral activity merely suggests a specific serotype.

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Effects of Mineral Deficiencies on Amino Acid Utilization. Critical Role of Potassium and Phosphorus. (20278)

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The acute metabolic disturbance which attends the simultaneous deficiencies of protein and potassium has been strikingly demonstrated by Cannon, Frazier and Hughes(1,2). Their studies show that potassium deficiency limits protein regeneration and that the rate of amino acid utilization under certain dietary circumstances may be proportional to the dietary adequacy of potassium. Part of the University of Chicago study was carried out with Aminosol,* a commercial fibrin hydrolysate, known to be almost devoid of potassium. Fibrin itself is low in all inorganic elements and has been used to advantage in designing a low phosphorus diet(3). In preparation of Aminosol, the fibrin is well washed prior to hydrolysis. Particular attention is paid to avoidance of sodium throughout manufacture. The nutritive balance of essential amino acids in this tryptophan fortified hydrolysate of fibrin is favorable, as demonstrated by the low nitrogen input required to bring about rapid protein regeneration in dogs(4), and rats(5,6). Daily requirements of the rat for some of the important mineral elements have been reported by Farris and Griffith(7) as follows: potassium 8-15 mg, calcium 40-50 mg, phosphorus 35-45 mg, and magnesium 1 mg. Twenty ml of the 5% fibrin hydrolysate, an amount which supplies amino acids for rapid recovery in protein-depleted rats, provided only about 0.1 mg of potassium, and not more than 0.02 mg of calcium, phosphorus or magnesium per rat day.

The present studies were designed to determine the influence of deficiencies of the above four mineral elements and of sodium on the response of protein-depleted rats to the fibrin hydrolysate.

Experimental. The non-protein diet, NP 11, used in these studies was essentially as before(5), except that it contained a higher proportion of fat. Also a change was made to Jones and Foster(3) salt mixture, which is well adapted to this type study. The composition is: sucrose 40, dextrin 30, lard 18, corn oil 3, agar 2.5, haliver oil 0.5, wheat germ oil 0.25, choline chloride 0.3 and salt mixture 4.0 g; thiamin · HCL 0.6, riboflavin 1.2, pyridoxine · HCl 0.6, calcium pantothenate 5.0, nicotinic acid 3.7, mixed tocopherols 2.5, folic acid 0.1 and inositol 5 mg per 100 g of diet.

TABLE I. Complete Salt Mixture and Mixtures Minus K, P, Ca, and Mg.

Salt mixture*	KH_2PO_4, %	$MgSO_4$, %	$CaCO_3$, %
Complete	38.9	5.7	38.1
Minus Ca	38.9	5.7	—
" Mg	38.9	—	38.1
" K	($NaH_2PO_4H_2O$—38.9)	5.7	38.1
" P	(KCl—21.2)	5.7	38.1

* Each salt mixture also contained NaCl 13.9, $FeSO_4 \cdot 7H_2O$ 2.7, $MnSO_4 \cdot 2H_2O$ 0.45, KI 0.071 $ZnSO_4$ 0.055, $CuSO_4 \cdot 5H_2O$ 0.048, and $CoCl_2$ $6H_2O$ 0.002%. Sucrose replaced the $CaCO_3$ and $MgSO_4$ when these were omitted.

1) *Deficiencies of K, P, Ca and Mg.* Male rats, 170-190 g, raised on the colony stock diet were depleted on non-protein diet in the usual way(5). Following drink trial and redepletion, five uniform groups of 6 rats each were formed. All rats were allotted 18.5 ml of 5% fibrin hydrolysate daily to supply 120 mg N. As a positive control, one group was continued on the non-protein diet adequate in all nutrients except protein. The test groups were fed non-protein diet in which the salt mixture was altered to impose a different mineral deficiency on each of the 4 groups. Table I shows the composition of the salt mixture(3) used in the non-protein diet, together with the changes devised to eliminate potassium, phosphorus, calcium or magnesium individually. The salt mixtures were all used at 4% of the non-protein diet. The 5 groups were fed the 5 different diets *ad libitum* for 11 weeks. The results are summarized in Fig. 1.

Deficiency of calcium or magnesium is surprisingly well tolerated in the protein-depleted

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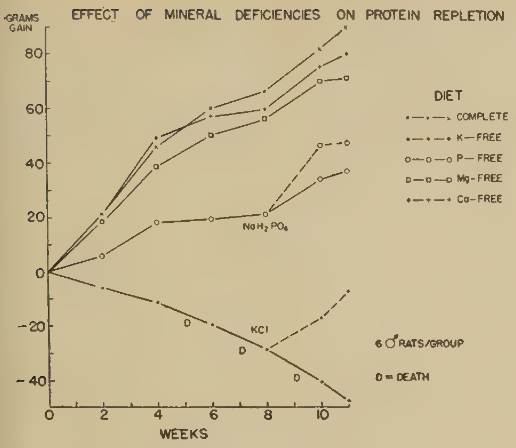

FIG. 1. Response of protein depleted rats to an intravenous fibrin hydrolysate (Aminosol) when fed *ad libitum* a non-protein diet complete in all mineral requirements, or deficient in the single elements potassium, phosphorus, magnesium or calcium.

rat, whereas lack of potassium becomes manifest very soon. Total deficiency of phosphorus places a less critical immediate stress on the organism than deficiency of potassium, but nevertheless manifests itself promptly in the decreased ability to build new tissue. A prompt weight response was observed when deficiencies of either potassium or phosphorus were corrected in individual rats. All of the potassium deficient rats became moribund and showed signs of hemorrhage.

2) *Quantitative requirement for K.* According to Cannon, *et al.*(2), 20 mg of KCl (10.5 mg K) per rat day is about the critical level of potassium needed to support full protein repletion. The Chicago studies were made using a solution of synthetic and purified amino acids practically devoid of potassium. We were interested to obtain the critical requirement for potassium in the 5% hydrolysate of fibrin which contained naturally only 2-3 μg K per cc. Five groups of 6 male rats each, which had been used twice previously for routine repletion assays, were fed stock colony diet for one week for equilibration and then depleted 17 days on non-protein diet. The weight loss during the depletion period was 20-25%. At the start of assay, the rats were transferred to the non-protein diet containing salt mixture minus potassium phosphate (Table I). One group was offered 36.5 ml of the fibrin hydrolysate equal to 0.24 g N per day with no added potassium. Other groups were offered the same volume of hydrolysate with graded amounts of KH_2PO_4 added to supply 1.5, 3.7, 7.5 and 15 mg potassium and 1.1, 2.7, 5.5 and 11 mg phosphorus respectively per day. The assay was carried for 12 days. Table II shows the average weight gain and per cent intake of allotment for each group.

The data, as regards potassium requirement, are in accord with those of Cannon, *et al.*(2) indicating that a full repletion response is not obtained with 7-8 mg K per rat day. The Chicago data suggest that 10.5 mg K per day as KCl is adequate. The 45.6 g response of our rats which received 14 mg K per rat day is considered about maximal at the 0.24 g N feeding level(5). The separate requirement for P will be considered in another report. Ten milliequivalents per liter of KH_2PO_4 in Aminosol appears to supply both K and P in adequate amount. Apparently 11 mg P per rat day is above the critical requirement level.

3) *Effect of sodium deficiency.* Comparison was made of the effects of sodium versus potassium deprivation. Non-protein diet was made up as free as possible of sodium. $MgCl_2 \cdot 6H_2O$ was used in the salt mixture in place of $MgSO_4$ and NaCl. The amount used provided twice as much Mg and 85% as much Cl as the regular salt mixture (Table I). KI was used to replace NaI. Potassium free salt mixture was prepared for this experiment by substituting calcium glycerophosphate for KH_2PO_4. This avoided the excess of sodium which had prevailed in the K-free diet in the first experiment (Table I). The amount of calcium glycerophosphate used provided 65% as much P and 150% as much Ca as the normal salt mixture. These changes did not significantly alter the concentrations of other ions in the diet. Three groups of 5 standard protein-depleted male rats were formed. Aminosol 5% with dextrose 5%, U.S.P. (Lot 617-5907R) was fed at a level of 18 ml to provide 120 mg N per rat day. This supplied only 3.2 mg of sodium per rat day, whereas Farris and Griffith(7) cited 40 mg Na per day as optimal. Intake of the

TABLE II. Effect of Adequacy of Potassium and Phosphorus, as KH_2PO_4, on Amino Acid Utilization by Protein-Depleted Rats.

KH_2PO_4, mQv, added/liter Aminosol*	K supplied/ rat day, mg	Intake of allotment, %	Avg 12 day wt gain and stand. error, g
0	.07	81	4.3 ± 3.3
1	1.4	90	3.6 ± 2.7
2.5	3.5	93	16 ± 2.7
5.0	7	100	27.6 ± 4.4
10.0	14	100	45.6 ± 3.0

* 36.5 ml of Aminosol, 5%, with dextrose, 5%, (serial 615-5914) fed/rat day with KH_2PO_4 additions as shown. Six male rats/group.

TABLE III. Comparison of Sodium and Potassium Deprivation on Ability of Protein-Depleted Rats to Use Amino Acids.

Deficiency in non-protein diet	Avg 12 day wt gain and stand. error, g
Control-complete	19.4 ± 1.5
Na	24.2 ± 3.1
K	4.0 ± 2.6

18.3 ml Aminosol, 5% in dextrose, 5% supplied 3.2 mg sodium and .04 mg potassium/rat day to all groups. The full supplement was consumed by all rats throughout.

daily allotment was complete for all rats throughout the 12-day assay. Results of the test shown in Table III indicated that sodium withdrawal does not immediately alter utilization of amino acids.

Discussion. A histological study of the heart lesions in protein depleted potassium deficient rats was made by French(8) as a corollary to the nutritional studies of Cannon, *et al.*(2). Myocardial lesions appeared in less than 12 days in the protein depleted animal when a diet deficient only in potassium was supplied. Potassium, supplied following initial deprivation, induced a rapid arrest of the progress of the heart lesions. The least severely damaged muscle fibers appeared to recover in part with compensatory hypertrophy and condensation of the surviving tissue.

The clinical syndrome characterized by potassium deficit and the strategic use of potassium salts to correct potassium imbalance has attracted much recent attention. Eliel, Pearson and White(9) have recently described the use of potassium phosphate supplementation in conjunction with complete parenteral feeding in postoperative patients. Such therapy receives strong theoretical support in our studies. Indeed, judging from the results of our studies, one would need to supply among the major mineral elements only potassium and phosphorus to meet short-term metabolic needs of the surgical patient. Phosphorus plays a less critical role, but its practical and theoretical significance is clearly apparent. Requirement for sodium, magnesium and calcium were apparently met by body stores of the rat for as long as 12 days under the conditions of our experiments.

The recent report of Menaker and Kleiner (10), which appeared after the completion of our studies, attaches more significance to magnesium deprivation than was apparent in our results. These authors indicated that magnesium deficiency did not result in any anatomical or physiological signs of deficiency, other than decrease in appetite and weight gain. Nevertheless they concluded "the unfavorable effect of magnesium deficiency on the synthesis of protein appears to be approximately as great as that reported for potassium deficiency." Although it is not our intention to minimize the relative importance of magnesium, it would seem from both our results and those of Cannon, *et al.*(2) that potassium is more critical. The point is an important one requiring considerable study. It would be impractical to incorporate many mineral elements into amino acid mixtures for intravenous use. Incorporation of potassium in commercial protein hydrolysates is quite practical and is, we believe, well founded nutritionally.

Various considerations suggested that a level of 10 mQv of potassium per liter of 5% protein hydrolysate would supply just enough of this element to support full utilization of amino acids. On the other hand, this level of potassium would offer no danger of inducing hyperkalemia. During fasting N and K are excreted in the ratio 10 to 1, the approximate ratio of these elements in muscle. The addition of 10 mQv K per liter provides a ratio of N to K in Aminosol of approximately 17 to 1, clearly a safe ratio. This allows for separate therapy with balanced electrolyte solutions, including additional K if desired.

Much more work is needed to establish how best to supply total nutriment parenterally. Part of the problem involves how best to combine amino acids, minerals and calories in the relatively small volumes of liquid which can be given parenterally. At the present stage of development it is generally possible to provide only those nutrients for recuperation to the point where full oral intake is resumed. Potassium appears to fit into this special group of nutrients.

Summary. 1. Results of this study point to the critical role of potassium in amino acid utilization following protein depletion. Rats depleted only of protein failed completely, even when offered a complete source of amino acids, if at the same time potassium was withheld. If phosphorus was withheld during the repletion period, the rate of recovery was lessened, but complete failure and deaths did not result as with potassium deprivation. Deprivation of sodium, calcium or magnesium did not appear to have immediate limiting effects on the appetite of protein depleted rats for a complete amino acid source, or on their ability to recover lost weight. 2. Fourteen mg of potassium per rat day appeared to meet the requirement for this element. Addition of 10 mQv of potassium per liter to an intravenous protein hydrolysate appears to supply an effective, safe ratio of potassium to amino acid nitrogen.

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Isolation and Purification of Plasma Thromboplastin: Its Physico-Chemical and Biologic Properties.* (20279)

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In the course of studies on the isolation of various platelet and plasma factors active in the process of blood coagulation the observation was made that prothrombin and stable factor cannot be adsorbed on $BaSO_4$ from citrated plasma(1,2). A saline eluate from the adsorbent, however, contained a factor able to shorten considerably the clotting time of recalcified human plasma and increase markedly the prothrombin utilization of native platelet-poor human plasma. This article describes some of the properties and the biologic activity of this factor which is tentatively identified as "plasma thromboplastin" (PT).

Materials and methods. (a) *Separation of PT from other coagulation factors.* Blood was collected from healthy individuals in Silicone† coated syringes and through Arquad 2-C‡ coated 19-gauge needles using one-tenth volume sodium citrate 0.2 M as the anticoagulant. Platelet-rich plasma was separated

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† General Electric Dri-Film 9987.

‡ Available from Armour Co., North Chicago, Ill.

by centrifugation at 1,000 rpm for 15 minutes at 4°C and transferred to glass test tubes. These were kept for 30 minutes at 37°C and then centrifuged at 4,000 rpm for 30 minutes at 4°C to remove the remaining formed elements. The supernatant was adsorbed with $BaSO_4$ (analytical grade) (50 mg/ml). Prothrombin, fibrinogen, labile factor (proaccelerin, accelerin, PPCF, plasma ac-globulin) and stable factor (proconvertin, convertin, SPCA, factor VII) were found in practically unmodified concentration and activity in the supernatant plasma. The $BaSO_4$ was washed thoroughly twice with distilled water at 4°C; finally an eluate was obtained by treatment with 0.85% NaCl solution (or sodium citrate 0.2 M) equal in volume to 1/10 that of the original plasma. The eluate was free of known coagulation factors and contained a highly potent preparation of PT. Optimum elution was obtained at pH 6.3 and 37°C. Maximum activity was obtained after 15 to 20 minutes incubation in glass. (b) *Further purification of PT.* The eluate containing PT was again adsorbed on $BaSO_4$ and the PT eluted with 0.85% NaCl solution. After acidification to pH 5.0 with bubbling CO_2 at 5°C a precipitate formed which was separated by centrifugation and found inactive. The supernatant yielded a white precipitate after 25% saturation with $(NH_4)_2 SO_4$. The precipitate was redissolved to the original volume of the eluate with 0.85% NaCl solution and dialyzed against 0.85% NaCl at 5°C for 24 hours. The final product represented a highly purified preparation of PT, with an average of 20 units (as defined below) per mg of N. (c) *Assay of PT.* The assay of PT activity was carried out as follows: Blood was collected in Silicone-coated glassware with the double-syringe technic(3) through Arquad 2-C coated needles without anticoagulant. It was centrifuged immediately at 4,000 rpm for 30 minutes. The "native" plasma contained approximately one to 2 platelets/cu mm and would only clot after several hours when incubated at 37°C in Silicone-coated test tubes. The prothrombin activity of its serum, as determined with a one-stage procedure(4) would be as high or higher than that of the original plasma. 0.1 ml of the PT eluate obtained as described in (a) was added to 0.9 ml of platelet-poor native plasma in glass test tubes. Clotting occurred promptly. One hour later the prothrombin activity of the serum was determined. The prothrombin time obtained was translated into percentage of prothrombin activity by the use of a dilution curve. This was prepared by the following technic. A potent preparation of PT eluate was diluted from 100 to 10% with 0.85% NaCl solution. 0.1 ml of each dilution was added to 0.9 ml of platelet-poor native human plasma. The prothrombin time of the serum from each mixture was then obtained one hour after clotting. The actual times were then plotted against dilution on semi-logarithmic graph paper. Activity of PT was calculated as the difference between 100 and the per cent activity of the serum. Thus, if serum exhibited a prothrombin activity of 40% the potency of the PT added to the native plasma would be (100–40) 60%. (d) *Unit of PT.* With the availability of a purified preparation, a unit of PT was tentatively established. This is "the amount of material which added in the volume of 0.1 ml to 0.9 ml of platelet-poor native human plasma will halve the prothrombin activity of the resulting serum one hour after the completion of clotting as measured by the one-stage prothrombin consumption test." As already stated, most of the purified preparations contained approximately 20 units of PT per mg of N.

Results. (a) *Activity of PT.* As indicated in Table I, PT was able to shorten markedly the clotting time of native plasma and of fresh normal human recalcified oxalated plasma. It normalized the clotting time, if prolonged, and the prothrombin utilization during clotting of native normal platelet-poor plasma (collected in Silicone) and plasma of patients with known hemophilia, thrombocytopenic purpura, and thrombasthenia. On the other hand, PT was unable to correct the delayed prothrombin time of plasma from patients treated with high doses of Dicumarol (showing marked deficiency of prothrombin and stable factor). Also, the delayed prothrombin time of stored human plasma (depleted of labile factor) was not affected. These results were confirmed by experiments in which PT

TABLE I. Activity of Plasma Thromboplastin.*

	Clotting time, min.		Prothrombin activity of serum, %	
	Before adding PT	After adding PT	Before adding PT	After adding PT
1. Native human plasma	5′15″	2′30″	12.5	0
2. ” platelet-free human plasma	90′	7′15″	95	0
3. Normal human oxalated plasma (recalcified)	2′15″	42″	—	—
4. Hemophilic plasma	29′	12′	100	4.5
5. Thrombocytopenic plasma	6′	3′	82	0
6. Plasma from patient with thrombastenia	—	—	58	2
	Prothrombin activity of plasma, %		Labile factor activity of plasma, %	
	Before adding PT	After adding PT	Before adding PT	After adding PT
7. Plasma of Dicumarolized patients	12	11	—	—
8. Stored human plasma	2.5	2.5	10.5	12

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* In each instance, PT eluate was added to various plasmas in 1/10 (equivalent to the amount of PT thromboplastin in 1 ml of plasma, since purified preparation is concentrated 10 times). The prothrombin activity of serum was determined 1 hr after completion of clotting. Each instance represents a typical experiment.

TABLE II. Activity of Plasma Thromboplastin from Various Citrated Plasmas, Platelets and Serum.*

	Prothrombin activity of serum, %	Plasma thromboplastin activity, %
1. Normal platelet-rich human plasma	1.5	98.5
2. ” ” ” ” ” (incubated in Silicone)	75.5	24.5
3. ” ” -poor ” ”	82.0	18.0
4. Suspension of washed platelets†	86.0	14.0
5. ” ” ” ” + platelet-poor plasma	29.0	71.0
6. ” ” ” , ether treated platelets + platelet-poor plasma	91.5	8.5
7. Normal human serum, shortly after coagulation	91.5	8.5
8. Plasma from heparinized patients	22.0	78.0
9. ” ” Dicumarolized patients (plasma prothrombin activity 9%)	1.0	99.0
10. Hemophilic plasma	85.0	15.0
11. Thrombocytopenic plasma	80.5	19.5
12. Hemophilic + thrombocytopenic plasma	9.5	90.5

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* In each instance 1/10 ml of purified PT obtained from plasmas, platelets or serum as described was added to 9/10 ml of platelet-poor fresh native human plasma; mixture allowed to clot and prothrombin time of serum determined 1 hr after completion of clotting. Each instance represents a typical experiment.

† Platelets were resuspended, after washing, in 1/3 volume saline; ground with $BaSO_4$ and the $BaSO_4$ eluted with saline solution.

eluates were prepared from plasma of various patients (Table II). Fully active eluates were obtained not only, as expected, from normal citrated plasma but also from plasma of severely heparinized or Dicumarolized patients. Human plasma stored for one week at 4°C exhibited only 15% labile factor activity but 50% plasma thromboplastin activity. On the other hand, no or minimal PT was obtained from plasma of severe hemophiliacs and patients with thrombocytopenic purpura; from isolated and washed platelets; from platelet-poor and platelet-free plasma, and from platelet-rich plasma carefully collected and kept in Silicone-coated surfaces. When thrombocytopenic and hemophilic plasmas; and isolated platelets and platelet-poor plasma were mixed, an eluate of normal PT activity was obtained. This did not occur when platelets were treated with ether prior to incubation with plasma. Eluates from serum immediately and at progressive intervals after clotting were found to contain practically no PT activity.

TABLE III. Properties of Plasma Thromboplastin.

1. Stable at 56°C for 30 min.
2. Fully active after 1 wk storage at –20°C; 50% active after 1 wk storage at 4°C; practically inactive after 3 wk storage at 4°C.
3. Not dialyzed against 0.85% NaCl solution for 24 hr.
4. Not sedimented after centrifugation at 40000 RPM for 1 hr.
5. Absorption curve shows maximum optical density at 278 μ.
6. Adsorbed on tri-calcium phosphate gel and barium sulfate from citrated plasma; eluted from it by 0.85% NaCl solution or 0.2 molar sodium citrate; maximal elution at pH 6.3, and 37°C.
7. Absorbed on 20%, 30% and 50% asbestos filters.
8. Completely precipitated from solution by 25% saturation with ammonium sulfate.

(b) *Properties of PT* (Table III). Samples of a saline eluate of Plasma Thromboplastin were still fully active after one week of storage at —20°C; samples stored at 4°C showed a loss of 50% activity after one week and total loss after 3 weeks. PT activity was relatively unimpaired after 30 minutes incubation at 56°C and after dialysis against 0.85% NaCl solution for 24 hours. Centrifugation of the eluate at 40,000 rpm for one hour failed to produce any precipitation and the upper layer was fully active. After centrifugation at 40,000 rpm for one hour maximal PT activity was obtained from the uppermost layer of plasma. A purified eluate showed maximum absorption at 278 μ (Fig. 1). Further chemical and electrophoretic data are being obtained. Asbestos filters 20%, 30%, and 50% absorbed PT completely. This, as already mentioned, could be precipitated from solution by 25% saturation with $(NH_4)_2SO_4$.

(c) *In vivo effects of PT.* PT was administered to 4 patients with thromboplastin-deficiency states, 3 with "amegakaryocytic" thrombocytopenic purpura and one with severe hemophilia. PT from 4 pints of freshly collected blood was prepared as described in the section on methods (a) and injected intravenously in a total volume of approximately 150 ml in 0.85% NaCl solution over a period of 25 minutes. There was no change in pulse rate, body temperature or blood pressure, nor did the patients complain of any subjective symptoms. In one of the patients receiving 2 injections at 15 days interval there was, likewise, no anaphylactoid reaction to the second administration.

In the 3 thrombocytopenic patients the prothrombin utilization during clotting was significantly increased for at least 8 hours following the injection (Fig. 2) and the clotting time of recalcified plasma slightly shortened, while no important change in the con-

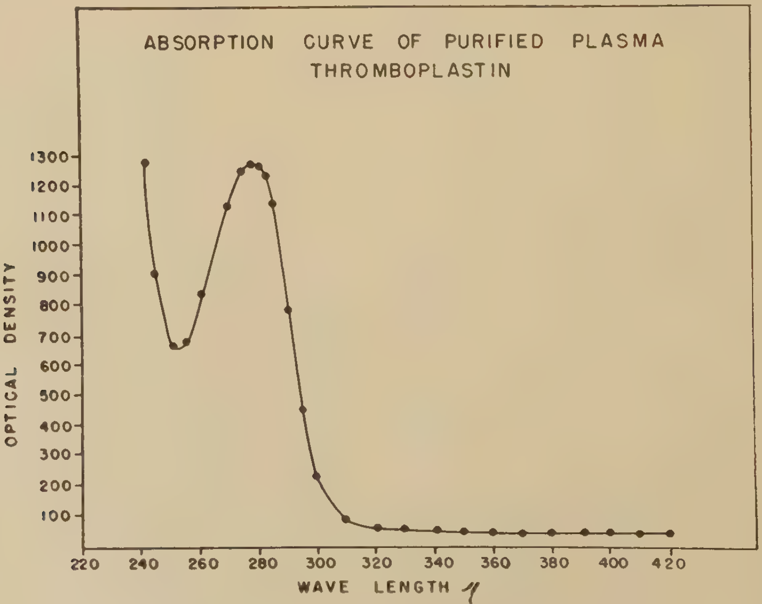

FIG. 1. Absorption curve of purified plasma thromboplastin.

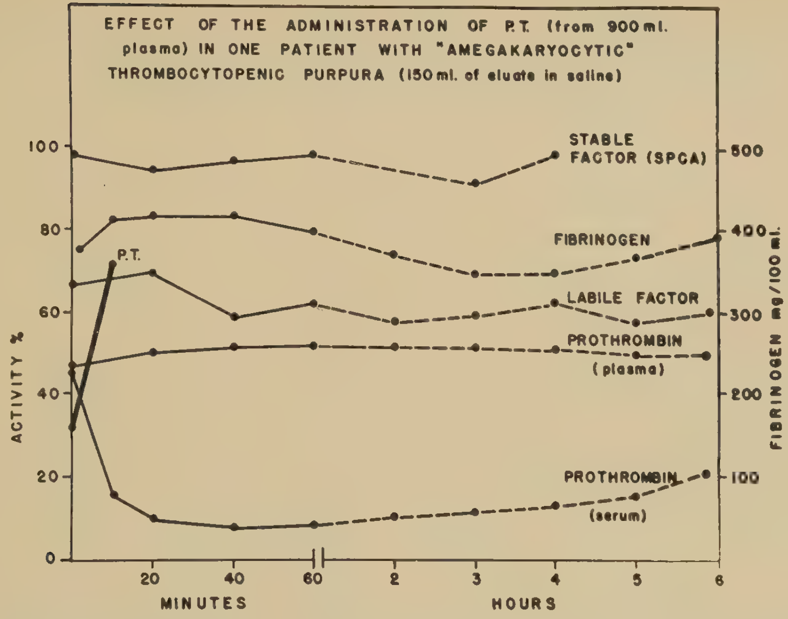

FIG. 2. Effect of administration of "plasma thromboplastin" (from 900 ml of plasma) in one patient with "Amegakaryocytic" thrombocytopenic purpura (150 ml of eluate in saline.

centration or activity of plasma prothrombin, labile factor, stable factor or fibrinogen was noted. The prolonged bleeding time and positive tourniquet test were unaffected. The patient with hemophilia showed only minor and transient acceleration in the clotting time and increase in the prothrombin utilization immediately after the administration of PT. The patient, however, had received only 1/3 of the minimum amount of PT *in vivo* needed to correct his hemorrhagic defect *in vitro* and therefore, possibly, an insufficient amount to cause any changes. The platelet count was halved immediately after infusion but returned to the original level within one hour.

Discussion. Physico-chemical properties and biologic activity differentiate PT from other known coagulation factors. The development of this agent requires the presence of at least one or more platelet and plasma factors and of a foreign surface. This makes it unlikely that PT represents an independent coagulation factor. It is generally conceded that the first stage of blood coagulation consists in the activation of thromboplastin following shedding of blood. It is likely that PT represents the product of this preliminary phase of blood clotting. Calcium is not apparently needed for the evolution of PT which, however, requires the presence of a foreign surface. Whether such a surface activates a plasma component or causes the liberation of a platelet factor or both cannot be stated from the data available at this time.

Summary. 1. A factor (plasma thromboplastin) can be absorbed from citrated human plasma which shortens the clotting time of normal oxalated recalcified human plasma and increases the prothrombin utilization of platelet-poor hemophilic and thrombocytopenic plasma. This factor has been eluted and purified. An assay for its activity has been developed. A unit of activity, physico-chemical properties and biologic activity have been defined. 2. Plasma thromboplastin can be clearly differentiated from other known coagulation factors on the basis of its properties; its evolution requires one or more platelet and plasma factors and contact with a foreign surface. It probably represents the final product of the first phase of blood coagulation (activation of thromboplastin). Its administration has been proven to correct *in vivo* the defective prothrombin utilization of thromboplastin-deficiency states.

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Effect of Feeding Fat on Duration of Thiopental Anesthesia. (20280)

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Studies of the mechanism of thiopental detoxication have led to the conclusion that its short duration of action is due to its localization in the fat depots of the body, rather than to its rapid destruction(1). This conclusion implies that the amount of body fat greatly influences the duration of thiopental anesthesia. Hermann and Wood(2) successfully demonstrated this by showing that a 5 g % decrease in body fat resulted in a 100% increase in the duration of thiopental anesthesia. As a result of these findings it was deemed of interest to determine the effect of dietary fat on the duration of thiopental anesthesia.

Methods. A homogeneous group of non-starved albino white rats was divided into two groups with an equal number of both sexes in each group. Two hours before the injection of thiopental each member of one group was fed 2 cc of corn oil by stomach tube while the other group received nothing, other than their normal diet. Then 30 mg/kg of sodium thiopental* (10 mg/cc in isotonic saline) was injected into a tail vein. The anesthesia time was determined by the duration of the loss of the righting reflex, that is the time taken for the animals to roll over onto their feet from the supine position. The rats were not injected oftener than once every 14 days to avoid an acute thiopental tolerance (3).

Results. Throughout this experiment considerable difficulty was encountered because of a large day to day variation in anesthesia time, which cannot be satisfactorily explained. On some days a mean sleeping time of 76 minutes for the control group and 25 minutes for the oil group has been obtained; while on other days, using the same dose, a mean sleeping time of 22 minutes for the control group was obtained as opposed to 11 minutes for the oil group. The results tabulated in Table I Exp. A and B demonstrate this wide day-to-day variation, but also show that in spite of this daily variation there consistently remained a significant difference between the sleeping time of the control and the oil fed group. The data presented in Table I, Exp. C were obtained by processing the entire lot of 34 rats in one day to eliminate the day-to-day variation. It can be readily observed that a highly significant decrease in anesthesia time was induced by the oral administration of oil.

Discussion. A review of the work of Brodie *et al.*(4) suggests a possible explanation for this difference. Brodie has shown that following the administration of thiopental a shift in concentration occurs until an equilibrium is reached at which time the body fat contains about 15 times the concentration of thiopental of the plasma and other body tissues. Under the conditions of this experiment a chylomicronemia and hyperlipemia might be expected to occur following the ingestion of the oil. When the thiopental is injected it may be taken up by the chylomicrons, thus reducing its effective blood concentration and therefore decreasing its anesthetic action. This might be visualized as a partition of the thiopental between the lipid and aqueous phases of the body. The additional lipid supplied by the ingestion of the oil causing a reduction in the thiopental in the aqueous phase.

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* Pentothal (Abbott).

TABLE I. Effect of Feeding Corn Oil on Duration of Pentothal Anesthesia in Rats on 3 Different Days.

Exp.		No. animals	Mean anesthesia time	Stand. dev.	Probability Student t test
A	Oil	9	25	6.2	
	Control	9	76	38.9	
	Mean diff.		51		<.01
B	Oil	7	11	3.5	
	Control	10	22	6.1	
	Mean diff.		11		<.001
C	Oil	17	13	2.85	
	Control	17	30	15.6	
	Mean diff.		17		<.001

Perhaps the fact that the standard deviation of the oil fed group was lower than that for the control group is also of some significance. It was noticed throughout the experiment that the control group was much more erratic than the oil group. The wide variations in the sleeping time of the control group could be attributed to small variations in the percentage of body fat, which Hermann and Wood(2) demonstrated to have a marked effect on sleeping time. The relative uniformity of the sleeping time of the oil fed group could be attributed to the hyperlipemia which may cause a much greater shift in the thiopental equilibrium to the lipid phase so that small variations in body fat do not result in as marked variations in sleeping time.

Summary. 1. It has been shown in rats that ingestion of oil preliminary to thiopental anesthesia will decrease the anesthesia time by about 50%. 2. A suggested explanation for this action is the production of a hyperlipemia by the oil, with the resultant uptake of the circulating thiopental by the chylomicrons.

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Inhibition of Lysozyme by Heparin.* (20281)

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The function of lysozyme in various mammalian fluids and cells has never been known. The presence of the enzyme has been demonstrated by its lytic effect on various microorganisms(1-3), but a natural substrate in the tissues has not been found(4). Meyer(4) has emphasized certain characteristics which are common to lysozyme and hyaluronidase. Hyaluronidase is inhibited by heparin(5,6), and the inhibition has been assumed but not demonstrated to be competitive because of the chemical similarity of the inhibitor and hyaluronic acid. Similarly, inhibition of lysozyme by heparin and related polysulfonates has been shown(7), again without investigation of the nature of the inhibition. The present study establishes the fact that the

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* This work was supported in part by grants from the National Microbiological Institute of the National Institutes of the Public Health Service, the Duke University Research Council and the Baxter Laboratories, Inc.

inhibition of lysozyme by heparin does satisfy the usual criteria for competitive inhibition.

Experimental. One ml of a solution of crystalline egg lysozyme† containing 2.5 μg per ml was added to 1.5 ml of a suspension of a dried preparation of *M. lysodeikticus* (Bacto Lysozyme Substrate) contained in a 75 x 12 mm cuvette kept in a water bath at 37°C. The concentrations of substrate used were 180, 132, 84, 72, 60, and 48 mg %; a wider range was not feasible because of the method used. Turbidity (optical density) was read on the Coleman Junior Spectrophotometer(8) at 5-minute intervals for 20 minutes, and the reading was converted into mg % of unlysed bacteria by a calibration chart. A further correction was found to be necessary: completely lysed bacteria gave a reading very definitely greater than zero. To correct for this, suspensions of bacteria of each concentration used were treated with crystalline egg lysozyme in final concentration of 2 μg per ml (*i.e.,* twice the concentration used previously) for 2 hours to ensure complete lysis. A linear relationship with a coefficient p of value 0.097 was found between this reading and the initial concentration of substrate, and since the calibration curve was also linear in this region, a simple correction was possible. If a is the initial substrate concentration and x is the concentration lysed at t minutes, the concentration unlysed is a—(l-p)x. From this (a-x) was calculated. Values of a—(l-p)x below 12 mg % were rejected because in this range, errors in determinations are relatively large. Six experiments were run, and all results for the same interval and the same substrate concentration were averaged, since they did not differ appreciably from each other.

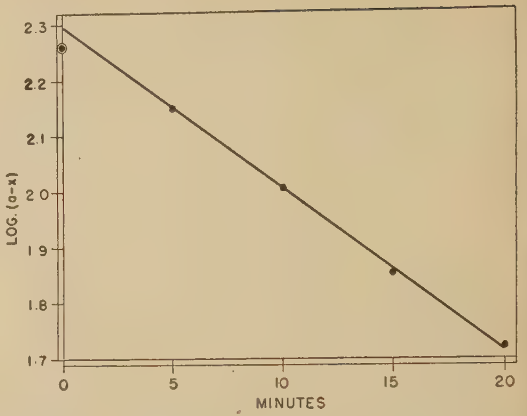

FIG. 1. Velocity of lysis of *M. lysodeikticus* by crystalline egg lysozyme. The logarithms of the mg % of unlysed bacteria are plotted against time. The straight line was drawn through the experimental points by the method of least squares, and the reaction is of the first order. Note that the point for zero time lies some distance from the line, indicating a lag, perhaps as a result of the time necessary for lysis.

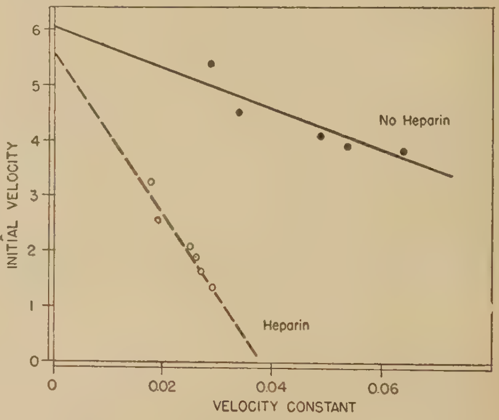

FIG. 2. Effect of heparin. The slope of the lines (drawn through the experimental points by the method of least squares) gives the Michaelis constant; the Y-intercept is V_{max}. Heparin greatly alters the slope without significantly affecting the Y-intercept.

When log (a-x) was plotted against time, it was seen that the points fell very close to a straight line, as in Fig. 1, indicating a first order reaction. Accordingly, data averaged as described were fitted to straight lines by the method of least squares. The slopes of these lines are the velocity constants. When these lines were extrapolated back to t = 0, they almost invariably indicated a higher initial substrate concentration than was actually used, or rather, a true zero time slightly later than the moment of mixing (see *e.g.,* Fig. 1, point ⊙). This perhaps indicates a slight lag between the combination of lysozyme with its substrate and the actual lysis of the bacterial cell. The velocity constants were plotted against the initial velocities obtained by multiplying the velocity constants by the initial substrate concentrations (Fig. 2).

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† Obtained through the courtesy of Armour and Co.

Again the points fell close to a straight line whose slope, determined by the usual least squares method, is equal to the Michaelis constant(9). Since the molecular weight of the true substrate, *i.e.*, the constituent of the bacterial cell containing the linkage upon which lysozyme acts, is unknown, the constant cannot be given in the usual units.

Simultaneously, a second series of determinations was run which differed from the first only in that the reaction mixture contained in addition heparin in a final concentration of 10 mg per ml. The initial velocity was again plotted against the velocity constant (Fig. 2), and it is obvious to the eye that the slope has changed. The change was found statistically to be highly significant. The intercept on the Y axis is V_{max}, and the difference in the two values for V_{max} is not significant. Inhibition by heparin of the action of lysozyme on *M. lysodeikticus* is therefore competitive(10).‡

Summary. The inhibition of lysozyme by heparin is shown by the usual criteria of the Michaelis-Menton formulation to be competitive in type.

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‡ Additional experiments have shown an inhibiting effect of large amounts of heparin on the lysozyme activity of lysates of human leukocytes also. No inhibition is evident at heparin concentrations in the range used in prior experiments for *in vitro* anticoagulation(8).

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Measurement of Complement Fixation in Small Volumes.* (20282)

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The applicability of the complement fixation reaction to the assay and immunological characterization of biological materials is frequently limited or entirely impractical because of the scarcity of the antigen or antiserum available. This is particularly the case in the virus field in which many useful studies have not been possible with the amounts of these materials needed for complement fixation as usually practiced. A specific problem encountered recently in this laboratory has stimulated an investigation of the adaptability of the standard reaction to quantitative work with small volumes. As will be described in this report, the results of the study indicate the promise of considerable success with the reaction in total volumes as small as 12 mm^3.

Materials and methods. The development of the procedure described here has depended principally on modifications of the standard method(1) for complement fixation necessary to overcome the technical difficulties introduced by the minute volumes employed. These have been circumvented in the main by the construction of apparatus eliminating the principal adverse factors. An initial prerequisite to use of the small volumes was

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† Scholar in Cancer Research of the American Cancer Society.

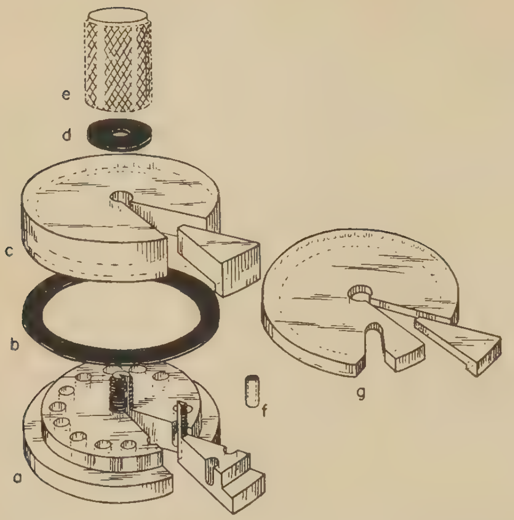

FIG. 1. The water-tight tube rack and the reaction tube used in micro complement fixation.

obviously the prevention of significant evaporation. This was accomplished with the apparatus shown in Fig. 1. Here there are illustrated the parts making up a rack or holder which carries the small reaction tubes during the preparation and incubation of the mixtures. The base, A, 2-1/16 in. in diameter, was turned from a lucite block 1/2 in. thick. At the periphery a shoulder was turned in the upper surface 1/4 in. deep and 3/16 in. wide. Holes, equally spaced and 13 in number, in the present instance, were drilled as indicated 0.177 in. in diameter and 0.280 in. deep. Numbers were engraved above alternate holes. At the center, a hole was drilled part way through the disc and threaded to take a 20-thread brass stud 1/4 in. in diameter and 11/16 in. long, which was cemented in with water proof glue. Item B is a rubber gasket 1/16 in. thick cut to fit over the shoulder of A. The lid, C, was turned also from a 1/2 in. plate of lucite and machined in the under surface to fit snugly over the shoulder of A. The inner surface of C and the upper surface of A were machined, and the depression in C was of a depth, slightly less than 3/16 in., such that when pressure was exerted on the gasket B, closed joints were made both at the contacting surfaces of A and C as well as at the surfaces of the gasket. A water-tight joint was formed by gasket D between the upper surface of C and the knurled knob E when the knob was drawn down on the stud. Another lid, G, was made for use during preparation of the mixtures. It was of the same diameter as A and, in the inferior surface, a depression was machined to fit the shoulder of A. The depth of this depression was only 3/32 in. deep. A notch was cut into the edge as indicated at G of a width and depth to allow access to a single reaction tube while all the others were covered. A hole was drilled in the center to allow free passage of the stud. At F is shown one of the reaction tubes which is a piece of pyrex glass tube 5/32 in. O.D. and about 3/32 in. I.D., closed in the flame at one end and cut of a length to bring the top of the tube exactly flush with the surface of A when the tube is put into one of the holes.

Measurement of the small volumes was effected easily, though somewhat tediously, by means of homemade pipets hand-drawn from standard pyrex tubing. The tubing was 3 mm O.D. (wall thickness 0.6 mm) for 1 and 2 lambda (λ) volumes, both in the same pipet; 4 mm O.D. (0.8 mm wall thickness) for 5 and 10 λ volumes, each in a separate pipet; 5 mm O.D. (0.8 mm wall thickness) for 20 λ volumes; and 8 mm O.D. (1.0 mm wall thickness) for 45 and 50 λ volumes, a separate pipet for each volume. The tips were left as fine as possible compatible with reasonable treatment on use, a point of some importance. A piece of plastic tube sealed with heat at one end made a good nipple for manipulating the fluid in the pipet. Commercial pipets could not be used because of the large tips. Calibration was accomplished with weighed volumes of mercury. Manipulation of the pipets was greatly facilitated through use of a jeweler's loupe. *Stirring of the mixtures* during incubation after the addition of red cells was of paramount importance; because of the very short distances in the small volumes, the cells settled out within a few minutes. After many trials with various methods it was possible to evade this difficulty by rotating the tubes in their containers in the device shown in Fig. 2, the construction of which is evident from the photograph. The size was determined by that of a small standard constant temperature

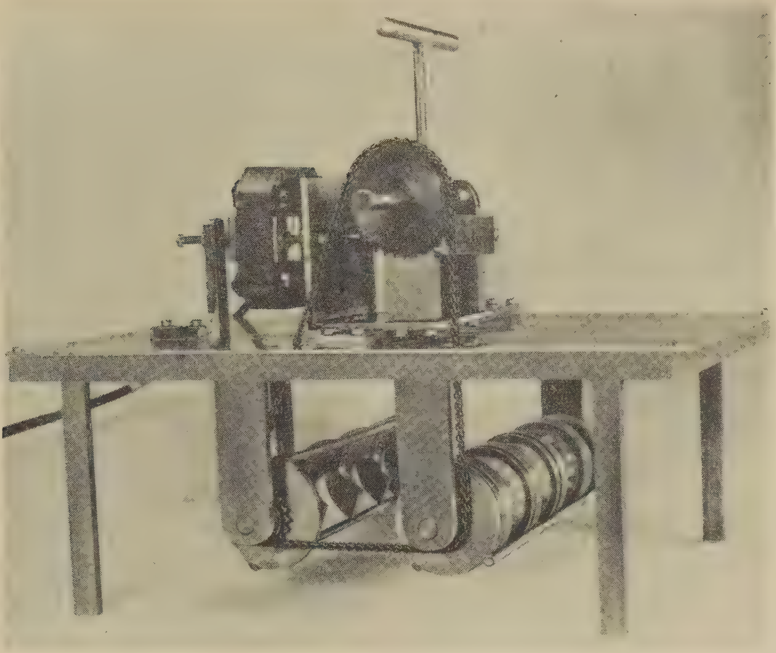

FIG. 2. Device for rotating the tube rack in the water bath.

water bath into which the device snugly fitted, resting on the four legs and effectively covering the bath. The speed of rotation of the tube containers, completely submerged in the water at 37°C, was about 100/min. In order to effect stirring, a single lead pellet from a 22 caliber long rifle shot cartridge was added to each tube. No adverse effects were observed with the use of lead; glass beads gave about the same results but tended to be caught in the meniscus of the liquid. It was found necessary to treat the glass tubes with silicone; otherwise, the cells gathered in the long angle made by the meniscus of the fluid and the tube wall.

Estimation of the *degree of hemolysis* was made by counting the number of red blood cells at the end of the reaction. These counts were made in duplicate in bright-line Neubauer counting chambers. It was convenient to use the 10 λ pipets for filling the chambers. Errors due to movement of the cover slip by touching it with the pipets were avoided by the use of clips made of thin-sheet phosphor bronze which held the slips firmly to the chamber. A thin sheet of aluminum was fastened to each end of the chamber to hold the latter above the stage, preventing displacement of the clips. In the standardization of the cell-counting procedure, a comparison was made of end points obtained by cell counts and estimation of hemolysis by hemoglobin determination in the standard way. The latter was done with reaction mixtures of 3.3 ml volumes, and hemoglobin was estimated in the Coleman spectrophotometer after sedimentation of the red cells. The percent hemolysis has been plotted on the probit scale in the graphs presented in this report. The sheep red blood cells, kindly provided by Mr. H. W. Craig, were obtained by the collection, washing and storage procedures described by Kabat and Mayer(1). A stock suspension, approximately 5% cells in Alsever's solution(1) stored in the refrigerator, could be used for several months. Cells for about 3-days work were washed with veronal-$MgCl_2$ buffer(2) (without $CaCl_2$) through 4 centrifugations immediately before each set of experiments, and cell concentration was determined by count. The cells were sensitized by the addition to the suspension of sufficient antisheep cell rabbit serum to yield 4 100% units of hemolysin. Complement was pur-

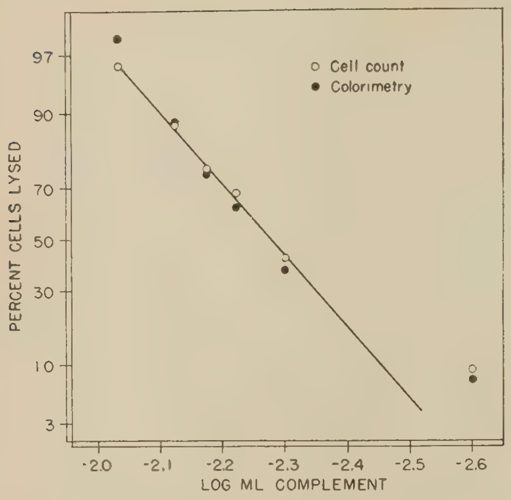

FIG. 3. Comparison of estimates of hemolysis in complement titration made by cell counts and colorimetry.

chased in the lyophilized state (Carworth Farms, New City, N. Y.), dried from carefully measurered 1.0 ml volume per ampoule from large volumes of guinea pig serum and reconstituted to the initial volume with the diluent supplied by the manufacturer. A fresh solution was made for each experiment, and its activity was of such uniformity that preliminary titration was not necessary. The test *antigen and antiserum*, obtained through the generosity of Dr. H. Piersma, Lederle Laboratories, were pneumococcus polysaccharide type VIII and the corresponding rabbit antiserum. A solution of the dry polysaccharide was prepared in a concentration of 0.759 mg per ml in veronal-$MgCl_2$ buffer and kept in the refrigerator without preservative. The fluid antiserum was parcelled in 10 λ volumes in melting point tubes (Kimble Glass, Toledo, Ohio), sealed at each end and stored in the frozen state at –20°C. At the time of its use, the tube and contents were heated at 56°C for 30 minutes, and a volume of 5 λ was diluted to appropriate concentrations with the veronal buffer.

Estimation of hemolysis by cell count and by colorimetry. As a preliminary to use of the small volumes, a series of studies was made to determine the correlation between the results of cell count and those of colorimetric measurement of the degree of hemolysis. The findings in one experiment are shown in Fig. 3. It is seen that the 50% end points of hemolysis estimated by the two methods were essentially identical, a finding encountered in a total of four experiments carried out in this way.

Titration of complement. A lengthy series of experiments was made in the simple titration of complement in order to develop the various technical procedures and to test the precision of the method finally adopted. The results of 5 such studies are given in the points of Fig. 4. These experiments were made over a period of 2 weeks; for each, a different ampoule of complement was diluted first to 1.0 ml, and further dilutions were made with the veronal buffer so that in the range expected to include the 50% hemolysis point, there were about 10 dilutions of complement available for study. These were spaced evenly with 6 dilutions covering the range of a 2-fold dilution. The reaction mixture consisted of 4 λ of buffer solution plus 5 λ of the appropriate dilution of complement. Mixing was effected by pumping buffer and complement solution several times with the pipet delivering the complement. These operations were carried out with all solutions chilled in ice water and with the tube rack standing on a cake of ice. The rack was sealed and immersed in a water bath at 37°C for 20 minutes. The rack was dried with a cloth, opened, and each tube

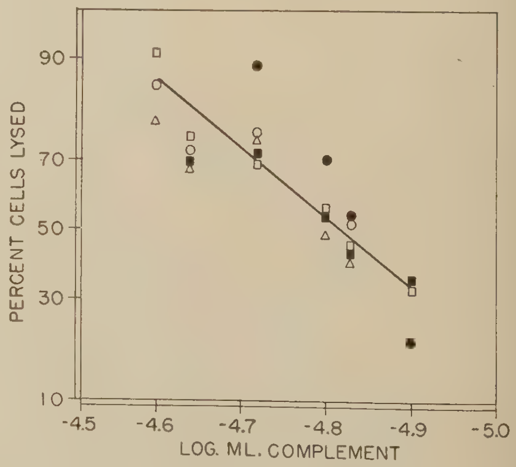

FIG. 4. The results of titration of separate batches of complement from the same pool over a period of 2 wk. The different symbols designate the findings in 5 different experiments.

received 2 λ of cell suspension containing a total of 20,000 red cells in the 2 λ volume (total volume of the reaction mixture was now 11 λ). Into each tube was placed the lead bead, and all were stirred with an inch-long piece of nichrome wire fixed in the handle of a bacteriological inoculating needle. The wire was washed and dried between tubes. After the rack was again sealed, it was placed in the rotator, submerged in water at 37°C and rotated for 30 minutes. Finally, the container was opened and, by inspection of the tubes, the range of hemolysis appropriate for cell counts was selected. To make the dilutions for counting, 20 λ volumes of buffer solution were measured into clean tubes and to each were added 5 λ of the respective, well-stirred reaction mixtures. The 25 λ volumes were thoroughly stirred by pumping with the pipet delivering the complement-red cell suspension into the diluent.

It is seen that the points of the different experiments shown in Fig. 4 are arranged in an apparently linear fashion and that those of 4 of the 5 experiments are a relatively close fit to the line drawn through the grouping by sight. The points of one experiment seem to lie along a line of a somewhat different slope from that through the others. Despite this difference in the slope, the position of the hypothetical linear relation at the level of the 50% point was very close to the analogous points in the other experiments. Analysis showed that the mean 50% unit of complement was 2.76×10^{-5} ml of the reconstituted material with a standard error of $\pm 0.23 \times 10^{-5}$ ml. It should be emphasized that these variations include not only possible differences in the properties of various specimens of reconstituted complement employed but also those to be expected in the repeated technical manipulations. It is notable also that this is a series of 5 successive experiments.

Fixation of complement by pneumococcus polysaccharide. A group of experiments were now made to learn the behavior of the system under those practical working conditions suitable to the study of complement fixation by a specific antigen and its antiserum. The procedure employed was modeled after that used by Mayer *et al.*(3). For this the pneumococcus polysaccharide, type VIII, and rabbit antipolysaccharide serum were employed. The relations of complement, antigen and antiserum and the results of 4 consecutive experiments are given in Table I. *Immediately before each experiment,* the stock polysaccharide solution described before was diluted further 1-400 with veronal buffer to provide the standard working solution for the day. With this preparation, different additional dilutions were made to yield, in 10 λ volumes, the amounts of antigen designated in the first column of Table I. Through this point the volumes were of the usual magnitude. The antiserum, after inactivation at 56°C, was diluted by adding 5 λ antiserum to 1.0 ml buffer solution. Reconstituted complement was diluted 1 part with 2 parts of buffer solution. The mixtures were now prepared by placing in the micro tubes in the chilled rack 10 λ of antiserum, 10 λ of complement and finally 10 λ of the various dilutions of antigen. Mixing was done with the pipet transferring the antigen. These mixtures, in the sealed racks, together with appropriate controls were incubated at 4°C for 16 hours. The racks were now opened, kept chilled and a series of twofold dilutions was made of each mixture, employing 10 λ volumes of mixture and buffer solution. The rack was again sealed and warmed in the water bath for 20 minutes at 37°C. To each tube there were now added 2 λ of red cell suspension (a total of 20,000 cells). A lead shot was put into each tube, the mixtures were

TABLE I. Fixation of Complement by Pneumococcus Type VIII Polysaccharide in Presence of Specific Rabbit Antiserum.

PN VIII in 30 λ reaction mixture ($\mu g \times 10^{-3}$)	50% units of complement fixed Experiments I	II	III	IV
3.80	47.8	54.9	>30	>48
1.73	31.2	38.0	>30	48.4
1.00	25.2	31.2	23.4	39.8
.70	11.1	25.2	20.4	23.7
.38	5.1	4.2	6.0	10.5
50% units complement in 30 λ reaction mixture	48.6	61.2	36.0	55.9
PN VIII antigen fixing 20 50% units complement —$\mu g \times 10^{-3}$	1.05	.77	.85	.62

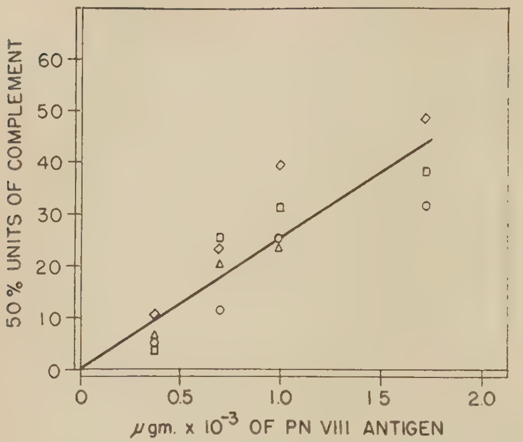

FIG. 5. Repeated estimates, in 4 experiments, of the complement fixing capacity of type VIII pneumococcus polysaccharide.

stirred with the wire, sealed again in the racks, and rotated for 30 minutes submerged in the water bath at 37°C. Appropriate dilutions of these mixtures were then used for counting the residual cells.

In Table I there are seen the various quantities of antigen employed with the constant volume of complement solution used in each experiment. The actual number of 50% point units of complement in this volume in each experiment was determined from the results obtained with the control mixture of inactivated immune serum and complement. The values in the body of the table indicate the number of 50% point units of complement fixed by each of the different quantities of antigen in the presence of a constant volume of antiserum. These results are shown graphically in the points of Fig. 5 which indicate, for practical purposes, a linear relationship between the number of 50% units of complement fixed and the quantity of antigen. From this relation there are calculated the amounts of antigen required to fix 20 50% units of complement. The 20-unit point was chosen for comparison since it lay about midway of the range of values. In the 4 experiments the amount of antigen required varied from 0.62 to 1.05 x 10^{-3} μg. The mean was thus 0.82 x 10^{-9} g with a standard error of the mean of 0.09 x 10^{-9}.

Discussion. The results of these experiments have shown that the complement fixation reaction can be employed on a scale sufficiently small to broaden greatly the range of reacting materials. The procedures described offer, in contrast with other micro methods(4), the advantage of quantitative data. Neither accuracy nor precision in a single experiment is as great as that obtainable with the use of larger amounts of antigen and antiserum; however, the exceedingly minute amounts of these materials needed for an experiment make possible the number of repetitions necessary for the required accuracy.

It was found highly advisable to employ the principle of complement fixation in mixtures of high concentrations of complement as used by Mayer *et al.*(3). In the small volumes, the loss of complement in the higher dilutions during the prolonged fixation period was both great and variable, and could not be eliminated by any means tried in this work. In other respects the technics, except for the greater tedium and care involved, differed little from the methods employing large volumes. The added difficulties are greatly overshadowed by ready accessibility to studies previously entirely impractical.

Summary. The equipment and the procedures have been devised for quantitative complement fixation in volumes of 12 mm³. The technic, reduced to a routine basis, is illustrated in the results obtained in the titration of complement and in the estimation of complement fixed by pneumococcus polysaccharide type VIII and its specific antiserum induced in the rabbit. With the procedures described the reaction can be carried out with antigen and antiserum in amounts of the order of 100-fold less than those required for the volumes usually employed, greatly extending the range for the study of material available only in small quantities.

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Certain Physical Properties of a Herpangina Strain and a Pleurodynia Strain of Coxsackie Virus. (20283)

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The present study was initiated to determine whether 2 members of the Coxsackie group of viruses which had been isolated originally from clinically different types of illness and which were known to be serologically distinct from one another possessed discernible differences in physical properties which might further serve to distinguish them. Strain No. 1816(1) (Huebner type H_3), which is immunologically identical with Dalldorf Group A, Type 10(2), was used as the representative Herpangina virus in the current work and strain No. 4520(3), which is identical with Dalldorf Group B, Type 3, was used as the Pleurodynia virus. Strain No. 1816 had been employed previously in electron microscopic and ultracentrifugal studies(4) and had been shown to be indistinguishable in size and sedimentation constant from another Herpangina (Group A) Coxsackie virus.

Several technics which have been reported elsewhere were adapted to the present study. These include the use of Attaclay in purification(5), estimation of sedimentation constant using concepts of pseudo-velocity and effective velocity(6), and pseudo-replication of agar blocks for electron microscope studies(7).

Materials and methods. Coxsackie virus, strain No. 1816, was propagated in 4-day-old mice and was prepared for examination in the electron microscope by the methods previously reported(4). The No. 4520 strain was propagated in 2-day-old mice and was purified in the same manner. The experiments with the separation cell required larger quantities of virus suspension than had previously been prepared, and a modification in the purification procedure was made. Following the general method of Hiatt *et al.*(5) the crude 10% suspension of infected mouse muscle was added to Attaclay SF* to give a 30% concentration of Attaclay. The mixture was shaken by hand and placed in a 2°-4°C refrigerator for a short time until it could be centrifuged at 3000 RPM for 10 minutes. The supernatant fluid was carried through the regular purification procedure starting with the ether extraction as described previously(4). *Infectivity* was determined by inoculating 0.04 ml amounts of several 10-fold dilutions of the material to be tested into each of eight 4-day-old mice. All mice were observed for 10 days after which the 50% end-point (LD_{50}) was calculated by the method of Reed and Muench (8). *Sedimentation* in the ultracentrifuge was recorded by both Schlieren and ultraviolet (2500 Å) absorption methods in a Model "E" Spinco machine. The separation cell(9) used in these experiments was made by replacing the regular analytical cell center section with one made of Lucite which had a dural barrier plate mounted in it. The Lucite center section was used without gaskets which facilitated assembly of the cell. All separation cell experiments were run at 17,250 RPM.

The concept of pseudo-velocity proposed by Epstein and Lauffer(6) has been used in the estimation of the sedimentation rate of the 4520 strain of virus since visible boundaries have not been observed with this agent. The pseudo-velocity is the ratio of the meniscus-to-barrier distance to the effective time of sedimentation. Since the sedimentation constant is defined as the velocity of the boundary per unit field of force at a given time and a given distance from the center of rotation of the rotor, we may make a calculation of the sedimentation constant from the pseudo-velocity rather than the measured velocity of a visible boundary. The formula for calculation is as follows: $S = \frac{\text{pseudo-velocity (cm/sec.)}}{\text{centrifugal force (dynes/g)}}$ where the centrifugal force is calculated at the distance of the barrier from the center of rotation. This value may then be corrected for the viscosity of the solution and the temperature of the experiment to give the value S_w^{20} usually reported. This method was used to calculate the sedimentation constant for the shortest times of sedimentation for which there was 10-fold or greater difference between the infectivity titer of the starting material and the material sampled from the top of the cell. The times of sedimentation used for the 4520 strain were chosen so that the boundary would not pass the barrier in certain runs but would be through the barrier in others. In the case of the strain 1816 separation cell experiments the visible boundary sedimentation constant was calculated as well as the sedimentation constant from the pseudo-velocity. Specimens were prepared for electron microscopy by *pseudo-replication* from agar blocks. Two per cent agar in distilled water was poured in petri dishes to a depth of 0.5 cm 12-24 hours before use. Small blocks 1 sq cm were cut from the agar and mounted on clean glass slides. A drop or two of virus suspension was allowed to evaporate to dryness in air on each block. The block was then flooded with a 0.5% solution of parlodian in amyl acetate. The glass slide was held vertically to drain off the excess parlodian. When the parlodian film was dry it was floated off on distilled water and 3 to 4 stainless steel screens were placed on each film which was then lifted from the water, film side up, on aluminum strips. The specimens were shadowed with chromium at arc tang ⅕. An RCA model EMU microscope with objective aperture and extended range lens was used.

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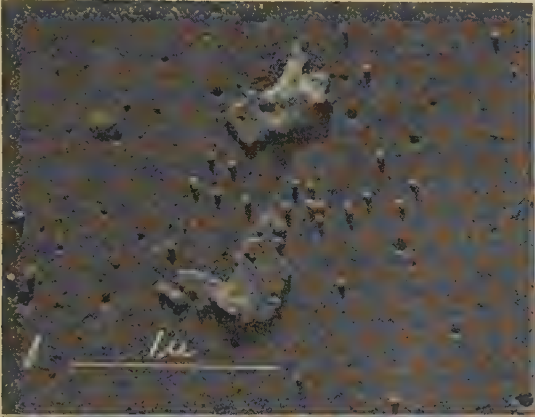
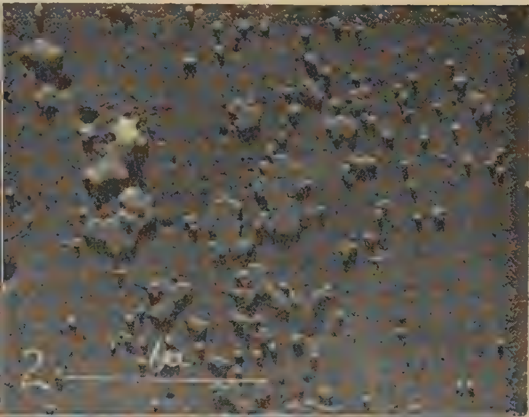
* Attapulgus Clay Co., Philadelphia, Pa.

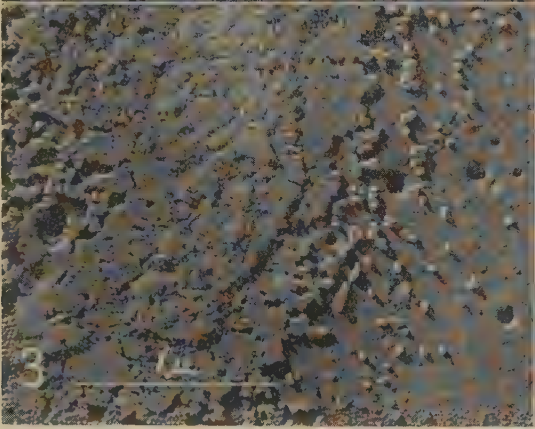
Results. Use of Attaclay. The use of Attaclay reduced the time required for processing a virus suspension for use in these experiments by about 30% since it eliminated overnight storage in the refrigerator and the freezing, thawing, and centrifugation used prior to the ether extraction in the purification procedure outlined previously(4). Two Group A and 2 Group B viruses were treated with Attaclay and the preparations used as antigens in complement-fixation tests and also in many of the separation cell ultracentrifuge experiments. The method was most effective when applied to suspensions having a nitrogen content of 0.7-1.0 mg/ml. The use of a 30% concentration of Attaclay was found to be optimal both for reduction of nitrogen content and for maintenance of complement-fixation and infectivity titers. For example with a preparation of 1816 virus the nitrogen was reduced from 2.67 mg/ml to 1.07 mg/ml with the infective titer remaining at 7.2. The infective titers were not significantly reduced, but the nitrogen was reduced about 60%. Furthermore, the use of Attaclay removed the pink color of the crude suspensions (due to hemoglobin) and also reduced to about one-third the amount of ether needed in the subsequent extraction of the supernate since adsorption on Attaclay apparently removes large quantities of lipoid material.

Electron microscopy. The electron micrographs of both strains of Coxsackie virus show spherical particles which are notably uniform. In both cases the particles have formed clusters which are hexagonal or diamond arrays. The diameter of the particles measured singly is approximately 37 mμ. Fig. 1 and Fig. 2 show the 4520 strain in small diamond-shaped regular arrays. The 1816 strain formed even larger clusters and, as is seen in Fig. 3 and Fig. 4, the groupings are hexagonal pseudo-crystals which have two or three layers or sheets of particles. The smaller particles of about 18 mμ diameter seen in the background are assumed to be a normal component of mouse muscle suspensions(4).

Ultracentrifugation. The sedimentation constant of the 1816 strain of Coxsackie has been determined previously(4) as being approximately 150S. Using the separation cell, a series of sedimentation runs was made with the 1816 strain in which the refractive index boundary was seen to approach and then pass through the cell barrier. The results of these experiments are shown in Table I. For these same runs the sedimentation constant has been calculated using the pseudo-velocity and the centrifugal force at the distance of the barrier from the center of rotation, *i.e.,* 6.723 cm. For estimation by this method the 2 shortest periods of centrifugation for which there was an infective titer reduction of 1 log or more in the fluid of the top compartment were used for calculating the sedimentation constant. In the experiments with the 1816







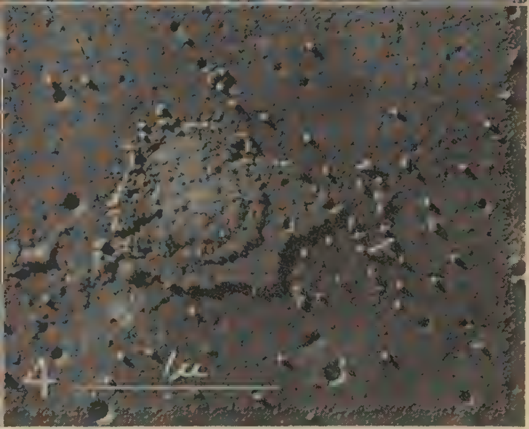


FIG. 1. Coxsackie virus—Strain 4520—pseudo replica from agar, chromium shadowed.
FIG. 2. Coxsackie virus—Strain 4520—pseudo replica from agar, chromium shadowed.
FIG. 3. Coxsackie virus—Strain 1816—pseudo replica from agar, chromium shadowed.
FIG. 4. Coxsackie virus—Strain 1816—pseudo replica from agar, chromium shadowed.

strain the average of the two sedimentation constants calculated from the pseudo-velocity and the infectivity difference is 167S compared with an average of 156S from the visible boundary calculation. Thus the sedimentation constant calculated indirectly is 7% greater than the observed value for the 1816 strain. Employing the pseudo-velocity technic on the 4520 strain provided a sedimentation constant of 175S. Assuming that this method of interpolation could be at least 10% in error, the range of sedimentation constant for the 4520 strain would be from 157S to 193S.

Discussion. The size and sedimentation constants of a Herpangina strain and a Pleurodynia strain of Coxsackie virus do not differ appreciably when estimated by procedures employing the electron microscope and the separation cell in the analytical ultracentrifuge. The electron micrographs of particles from both sources reveal uniformly spherical bodies. Using the concept of pseudo-velocity and the change in infectivity in the top compartment of the separation cell for estimating the sedimentation constant, there is a difference of less than 5% between the values for the two strains of virus. In the case of 1816 strain this method of estimation is some 7% higher than the sedimentation constant calculated from the visible boundary.

The application of pseudo-velocity used here differs from that of Epstein and Lauffer (6). In their experiments the pseudo-velocity was used to identify the visibly sedimenting particle as the infectious agent. The assumption in this study is that a significant change in infectivity in the separation cell

TABLE I. Data on Separation Cell Experiments with Coxsackie Viruses.

Coxsackie strain	Effective time of sedimentation, sec.	℄ to Meniscus, cm	Pseudo velocity, cm/sec.	Difference in titer starting mat'l and top of cell log	S_w^{20}* calc.	S_w^{20}† obs.
1816 (Group A)	600	5.93	1.32×10^{-3}	.1	524	144
	960	5.98	.777	.8	307	155
	1740	5.95	.444	1.5	171	153
	1680	6.05	.403	2.5	163	159
	1080	6.32	.374	2.1	152	
4520 (Group B)	540	6.00	1.35	.6	584	
	960	5.98	.777	.8	335	
	1920	5.90	.428	1.6	202	
	2400	5.96	.317	1.7	148	
	3000	5.95	.257	2.0	117	

* Using pseudo velocity and centrifugal force at barrier distance 6.723 cm.

† Calculated from visible boundary on photographic plates.

indicates that a boundary exists and has crossed the barrier. When this boundary is not visible the pseudo-velocity may be used to estimate the sedimentation constant of the infectious agent. The difficulty in preparing sufficiently concentrated preparations of relatively pure animal viruses to produce refractive index boundaries in the ultracentrifuge makes an indirect method based on biological measurements, such as infectivity, particularly desirable.

The electron micrographs presented in this study show the virus particles in uniform clusters. These formations have occurred only in those specimens made by pseudo-replication of agar blocks. Previous electron micrographs(4) of the 1816 strain, for example, were made from specimens prepared by placing a drop of suspension on parlodian coated screens and drying in air, and in no case did the particles form clusters. The present electron micrographs show clusters very similar to those previously seen in purified plant viruses(9). Further investigation of the technic including the effect of pH change in the agar and varied concentration of virus may reveal the optimum conditions for cluster formation.

Summary. 1. There is no appreciable difference between the particle diameters of the 2 Coxsackie viruses—Strain 1816 (Herpangina) and Strain 4520 (Pleurodynia). The diameter is approximately 37 mμ for single particles and about 28 mμ for particles measured in rows. 2. The sedimentation constants, estimated indirectly, are 167S for the 1816 strain and 175S for the 4520 strain. For the 1816 strain the visible boundary measurements give 153S in this series of experiments. 3. Attaclay is useful in the procedure for preparing partially purified virus from suspensions of infected mouse muscle. 4. The technic of pseudo-replication from agar blocks has led to electron micrographs showing the virus particles in orderly hexagonal arrays.

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Nucleic Acids of the Mammary Glands of Rats.* (20284)

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Two concepts of the relationship of nucleic acids to growth and activity of tissues suggested the present study of the growth and development of the mammary glands of rats. First of these is the concept that the desoxypentose nucleic acid (DNA) content of cell nuclei is constant for the various somatic cells of an organism(1,2). The second is that pentose nucleic acid (PNA) is closely related to protein synthesis(3,4).

The formation of the lobule-alveolar system in the mammary gland in early pregnancy is brought about by extension of the major ducts and rapid proliferation of the side branches and alveoli. The magnitude of cellular proliferation in this example of growth should be directly related to an increase in DNA in the total gland complex (mammary gland plus fatty pad). Analysis of PNA in the developing and lactating gland was of interest since the mammary gland is an organ highly specialized in the synthesis of fats, carbohydrates and proteins. Considering the DNA in the cells as constant then a survey of the ratio of PNA to DNA should give an evaluation of the condition of the cells in the growing and actively secreting mammary glands.

Materials and methods. Virgin, pregnant nulliparous and lactating primaparous albino rats were used in this study. The rats were bred at a weight of about 150-160 g. Some rats of this weight were killed and used as a virgin reference. Days of pregnancy in other rats were determined by examination of the size of the fetuses. Litters of lactating rats were reduced or increased to 5 in number. Young were removed from their mothers at 25 days of lactation and the latter killed 5 or more days later in order to obtain the gland undergoing involution. The animals were killed, rapidly skinned, and the 6 posterior glands (fatty pads) removed from the skin, pooled, weighed and frozen. The tissue from each rat was placed in a preweighed extraction thimble and lyophilized. The thimble and dried gland were weighed to determine the water content. The tissue was then extracted in a continuous extractor with hot alcohol for a period of 8-10 hours followed by extraction with ether for a similar period. The dry fat-extracted glands were weighed again to determine the fat-free weight. Each sample of glandular tissue representing the original 6 posterior glands of one female was ground to a fine powder in a Wiley mill. Fifty mg aliquots of the glandular powder were used for DNA and PNA determinations. Twenty-five mg aliquots were used for nitrogen determinations. DNA and PNA were extracted from the tissue sample by the procedure of Schneider(5) with the exclusion of the fat extraction step, the fat having already been extracted. The dry tissue powder was brought in contact with 2 ml of water in the cold for 2 hours in order to wet the tissue. The tissue was then extracted with 5 ml of cold 10% trichloroacetic acid (TCA), centrifuged and the supernatant discarded. The residue was extracted with 10 ml of hot 5% TCA twice for periods for 15 minutes each. This gave complete removal of substances giving color tests for DNA and PNA. The 5% TCA extracts were diluted to 25 ml and DNA determined on 3 ml aliquots by the diphenylamine reaction (6). PNA was determined on one ml aliquots by the orcinol reaction(7) modified by heating for one hour(8). Commercial DNA and PNA was repurified and used to prepare standard curves. The densities were read on a Beckman model DU Spectrophotometer. Nitrogen was determined by the micro-Kjeldahl procedure. No attempt was made to correct for the nitrogen content of milk present in the glands.

The values of DNA and PNA as determined were calculated for the total glandular ma-

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* Contribution from the Department of Dairy Husbandry, Mo. Agri. Exper. Station. Journal Series No. 1356. Approved by the Director.

† Postdoctoral Fellow of the National Cancer Institute.

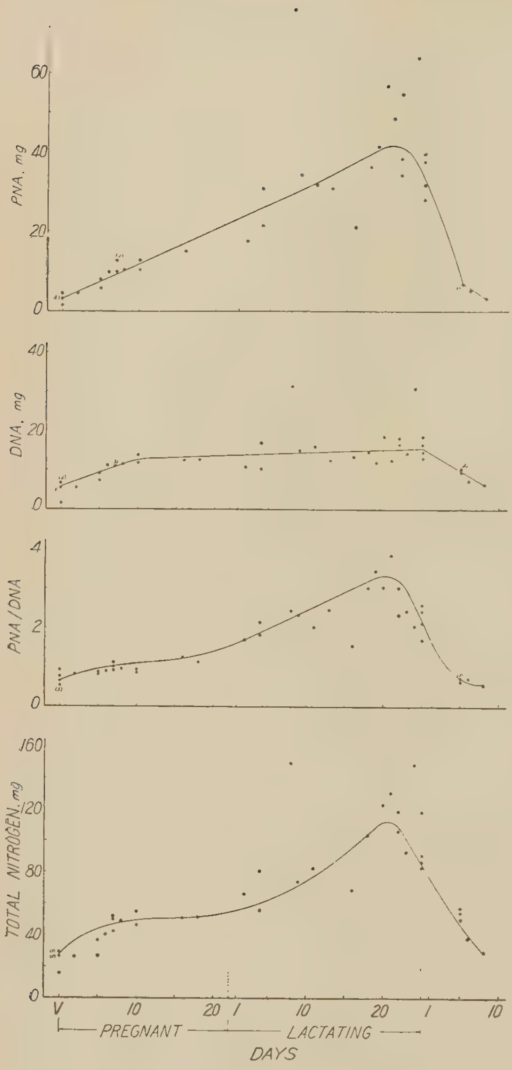

FIG. 1. PNA, DNA, PNA/DNA, and total nitrogen in the 6 posterior mammary glands of rats at different periods of pregnancy and lactation.

terial (fat free) taken from one female. The values obtained were plotted against days of pregnancy or lactation. The PNA-DNA ratio was calculated for each sample of glandular material and plotted as a function of the condition of the animal. Total nitrogen of the sample was calculated and plotted as above.

Experimental. In the virgin females the values of DNA, PNA, PNA/DNA and nitrogen were low signifying the undeveloped condition of the mammary glands. During the first half of pregnancy the DNA increased rapidly reaching a plateau at about mid pregnancy. From mid pregnancy to a point of maximum lactation the DNA in the glands increased only slightly.

The PNA rose parallel to DNA in the first half of pregnancy but continued to increase throughout lactation reaching a maximum value at 21-22 days. This increase in concentration of PNA above DNA is better shown by the increasing ratio of the 2 through pregnancy and lactation reaching a maximum at 21-22 days of lactation. Of interest here is the fact that glands represented by 8 and 24 days of lactation show values for DNA, PNA and nitrogen out of proportion to the other glands studied, yet the PNA-DNA ratio fits well with the values for the other glands.

The values for total nitrogen in general show the same trends as the PNA.

At involution the PNA sharply decreases in the mammary gland. The values of PNA, PNA-DNA and nitrogen are reduced in a manner approaching the values of the undeveloped gland.

Discussion. Reece and Warbritton(9) found that the mitotic activity of rat mammary glands was greatest at 10 days of pregnancy and low through lactation. Others (10,11) found that the epithelial tissue reached its maximum proliferation by mid pregnancy. The rise in DNA in the total gland in the first half of pregnancy agrees well with this histological data. The 2-fold increase in DNA from the virgin condition to mid pregnancy appears to be low for such a rapidly developing tissue. There are 2 points to be considered in this connection. One, that the increase in DNA represents a doubling of the total number of cells in the entire fatty pad. In the undeveloped gland the fat and connective tissue make up a major portion of the total fatty pad. Under the stimulus of pregnancy cell proliferation occurs predominantly in the epithelial tissues. A second consideration is that the virgin condition is represented by rats that may have been through several estrous cycles, hence the duct system of the mammary glands would be somewhat elaborated and the base level shown here would be greater than a base level that may be shown by castrated animals.

An increase in the PNA content of regenerating liver has been shown by several authors(12,13). However, in a study of the relation of nucleic acids to protein formation in the pancreas, Daly and Mirsky(14) failed to show any significant change in PNA content during the cycle of secretion. These authors object to the use of the mammary gland for studying the relationship of nucleic acids to protein synthesis on the basis that protein synthesis in the mammary gland depends on the formation of new cells. This objection is without foundation for the scarcity of mitosis in the lactating gland(15-17) indicates that in milk secretion there is little turnover of cells.

In the present study it is seen that the PNA increases to a maximum at 21-22 days indicating an average increase in PNA concentration per cell. In relation to this, the data of Brody and Nisbet(18) showed that some rats appeared to reach maximum milk production at 18-19 days of lactation while others did not reach maximum production in the 21-day period studied. Thus the PNA level in the mammary gland closely parallels the synthetic activity of the gland.

Nitrogen values of the mammary glands increased with the height of lactation reaching a maximum value at 20-22 days. A considerable portion of this nitrogen is undoubtedly represented by milk nitrogen, however, a part of the increase must represent hypertrophy of the alveolar cells. Although no attempt was made to calculate the nitrogen of the gland contributed by milk this could probably be done on dry tissue similarly as it was done on wet tissue by Folley and Greenbaum(19).

McShan and coworkers(20) demonstrated a rapid rise in PNA in the pigeon crop gland tissue under the influence of administered lactogenic hormone. The DNA increased initially but reached a plateau while the PNA continued to increase. This is similar to the results reported here for the normal growth and activity of the mammary glands of rats.

The downward trend in PNA at 25 days of lactation may be the result of voluntary weaning. Forced weaning at the 25th day of lactation is followed by a rapid decline in the PNA and the PNA-DNA ratio. This decline in involution is like the rapid fall in enzyme activity of rat mammary glands on involution as reported by Folley and Greenbaum(19).

Factors influencing the growth and maintenance of the mammary gland in a secretory condition are under investigation.

Summary. A study of the nucleic acid concentrations (DNA and PNA) in the developing and functioning mammary glands of rats is reported. DNA increases in the early part of pregnancy and increases only slightly in the lactating gland. PNA increases throughout pregnancy and continues to increase in the lactating gland reaching a maximum value at 21-22 days of lactation. Following enforced weaning the PNA content of the gland falls rapidly. These findings are discussed in regard to growth and function of the glands.

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Inhibition of Aldehyde Oxidation by Adrenergic and Adrenolytic Substances.* (20285)

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The metabolic disposal of epinephrine and related amines is usually attributed, in part at least, to the monoamine oxidase system. This system oxidizes amines to the corresponding aldehydes which then will be converted to the acid. The studies reported in this paper show that the enzyme present in guinea pig liver which converts aromatic aldehydes into the corresponding acids is inhibited reversibly by adrenergic amines and irreversibly by adrenergic blocking agents.

Methods. Enzyme activity was measured by means of an ultraviolet spectrophotometric method. Fig. 1 shows that salicylaldehyde, salicylic acid and saligenin (salicylalcohol) have distinct absorption peaks in the ultraviolet. The molar extinction coefficient of salicylaldehyde is 2300 at 325 mμ (the peak at 260 mμ was not used because of its proximity to the absorption zone of trichloracetic acid), that of salicylic acid 4450 at 303 mμ. These values are valid only for the present experimental conditions since the spectra are very sensitive to pH changes(1). The substrate used in all experiments was salicylaldehyde (Eastman-Kodak). The enzyme was prepared from guinea pig liver homogenized in 0.1 M phosphate buffer at pH 7.4. The

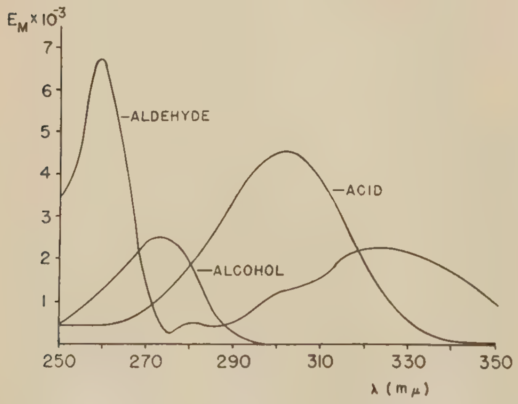

FIG. 1. Ultraviolet absorption spectrum of salicylaldehyde, salicylic acid and saligenin, in the presence of trichloracetic acid in proportions specified in the text. λ = wavelength in mμ; E_M = molar extinction coefficient.

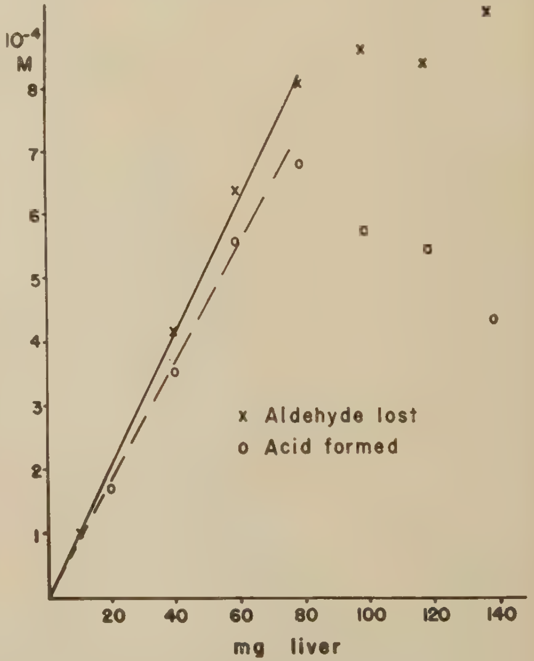

FIG. 2. Influence of enzyme concentration on disappearance of salicylaldehyde and formation of salicylic acid. Abscissa: enzyme concentration in mg of fresh liver per 3 ml of reaction mixture. Ordinate: aldehyde lost or acid formed in 10 min. The initial concentration of salicylaldehyde was 10^{-3} M in 3 ml.

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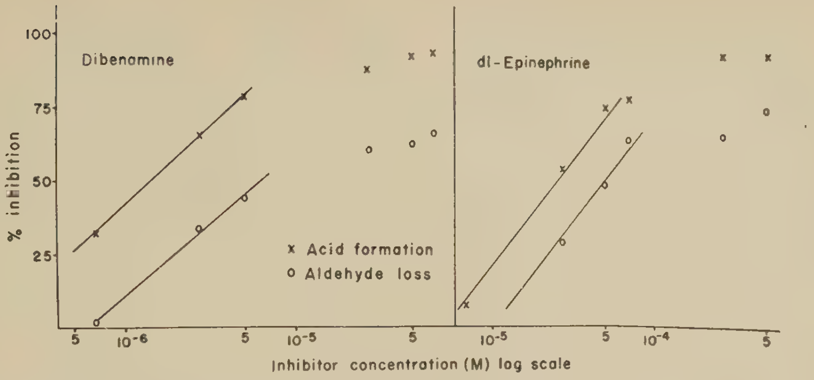

FIG. 3. Inhibition of aldehyde oxidation by Dibenamine (left) and epinephrine (right). Abscissa: molar concentration of inhibitor (log scale); ordinate: % inhibition of aldehyde loss (○) and acid formation (×).

cell debris was separated by centrifugation and the clear supernatant was made up with the buffer to correspond to 200 mg of fresh liver per ml. The reaction was started by adding the substrate to the enzyme solution. The total volume of the reaction mixture was 3 ml containing 0.4 ml of the enzyme solution, 1 μM of substrate and whatever inhibitor was being tested. The final buffer concentration was 0.067 M. In some experiments the enzyme and substrate concentrations were varied. After 10 minutes' incubation at 37.5°C the reaction was stopped by addition of 7 ml of 15% trichloracetic acid. The solution was left standing for at least one hour and filtered on hard paper (Whatman No. 50). When inhibitors were used, these were incubated with the enzyme solution for 15 minutes before addition of the substrate. The filtered solutions were read on the Beckman spectrophotometer at 303 and 325 mμ against a blank containing all the reagents except the substrate. From these readings the concentrations of salicylaldehyde and salicylic acid were calculated on the basis of the extinction coefficients given above. Since the aldehyde absorbs also at 303 mμ, a correction was made by means of its molar extinction coefficient at 300 mμ (1250). No saligenin formation could be detected in any of the experiments. Results are expressed in μM of aldehyde lost and acid formed during the reaction. All amines, unless otherwise stated, were used as hydrochlorides.†

Results. Fig. 2 shows that reaction velocity increases linearly with enzyme concentration up to about 80 mg of liver. It is seen that all the aldehyde destroyed is not converted into acid. The fate of this fraction of salicylaldehyde is unknown. It is likely that the oxidation goes beyond the stage of salicylic acid. This probably also occurs with high concentrations of enzyme.

Inhibition experiments were performed with 80 mg of liver extract. As seen in Fig. 3, the aldehyde loss cannot be entirely inhibited, no matter how high the concentration of inhibitor. Inhibition is therefore best expressed in terms of acid formation. Fractional inhibition is linearly related to the log of inhibitor concentration and the concentration of inhibitor required to cause 50% inhibition of acid formation is read from curves like those shown in Fig. 3.

Inhibition of the oxidation of salicylaldehyde by epinephrine and related amines is shown in Table I. The range of concentrations which cause 50% inhibition goes from 10^{-6} M to 10^{-3} M. One adrenergic amine, ephedrine, was found inactive. No inhibition was observed with the following aliphatic and aromatic amines: methylamine, diethylamine,

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† Grateful acknowledgement is made for generous supply of various compounds to Drs. R. K. Richards, L. W. Roth (Abbott Laboratories), F. G. Henderson (Lilly Research Laboratories), S. Fromer (Merck and Co.), R. W. Fleming (Parke, Davis and Co.), H. F. Schnizer (Sandoz Pharmaceuticals) and M. R. Nance (Smith, Kline and French Laboratories).

TABLE I. Inhibition of Aldehyde Oxidation by Adrenergic Amines.

Compound	50% inhibition (M conc.)
Epinine	1.6 × 10^{-6}
Adrenalone	7.3 × "
l-Epinephrine	1.4 × 10^{-5}
dl-Epinephrine	2.4 × "
Adrenochrome	2.5 × "
d-Epinephrine	3.7 × "
m-Synephrine	4 × "
5-Hydroxy-tryptamine creatinine sulfate (Serotonin)	4.3 × "
dl-Norepinephrine	5 × "
Ethylnorepinephrine	5 × "
Tryptamine	6 × "
p-Synephrine	1.3 × 10^{-4}
Tyramine	1.4 × "
p-Hydroxy-N-methylbenzedrine (Veritol)	1.5 × "
Isopropylnorepinephrine	10^{-3}

ethanolamine, benzylamine, methylmandelamine, 2-hydroxybenzylamine, histamine, acetylcholine and thyroxine. Two aromatic amino-acids, tyrosine and phenylalanine, were also inactive. Two deaminated derivatives of adrenergic amines, benzoylcarbinol and 3,4-dihydroxybenzoylcarbinol (derived from epinephrine) inhibited aldehyde oxidation in the concentration range of 10^{-4} M. The latter compounds were synthesized at this Institute by Mr. B. W. Turnquest.

Table II shows that oxidation of salicylaldehyde to salicylic acid is also inhibited by adrenergic blocking agents. All drugs of proved adrenolytic activity, with the notable exception of yohimbine, were found inhibitory for the reaction. The most potent inhibitors were Dibenamine and its oxyisopropyl derivative Dibenzyline. The ergot alkaloids and Priscol were less active followed by the benzodioxane derivative, 933F, and the weak adrenolytic agent diethylaminoethanol. Phenylmethylpiperazine and a few β-haloalkylamine derivatives were inactive at the concentration of 10^{-3} M; all have weak adrenolytic activity. Nitrogen mustard itself, which belongs to the same chemical series and which has no adrenolytic action, was found inactive on aldehyde oxidation.

Among the large number of other compounds tested, two groups deserve special mention: the aldehydes and antihistamine drugs. Three aliphatic aldehydes inhibited the oxidation of salicylaldehyde at varying concentrations: crotonaldehyde (3.2 x 10^{-5} M), butyraldehyde (4 x 10^{-5} M) and acetaldehyde (2.7 x 10^{-4} M). Formaldehyde and two aromatic compounds, benzaldehyde and anisaldehyde, were inactive.

Four antihistamine drugs were tested. Neoantergan (2-[2-dimethylaminoethyl(*p*-methoxybenzyl)amino]pyridine) was inactive but the other 3 proved potent inhibitors: Phenergan (10-(2-Dimethylamino-1-propyl)phenothiazine) at 7 x 10^{-6} M, Pyribenzamine (2-[Benzyl (2-Dimethylaminoethyl)] aminopyridine) at 2.3 x 10^{-5} M and Antergan (N',N'-Dimethyl - N - phenyl - N - benzylethylenediamine) at 3 x 10^{-5} M.

TABLE II. Inhibition of Aldehyde Oxidation by Adrenergic Blocking Agents.

Compound	50% inhibition (M conc.)
N-(2-chloroethyl)dibenzylamine (Dibenamine)	1.4 × 10^{-6}
N-(2-chloroethyl)phenoxyisopropyl-N-benzylamine (Dibenzyline, SKF 688A)	1.8 × "
N-ethyl-N(2-bromoethyl)-1-napthalenemethylamine (SY 28)	6.7 × "
N-ethyl-N(2 chloroethyl) benzhydrylamine (SY 2)	1.2 × 10^{-5}
Hydergine*	2.5 × "
N-methyl-N(2-chloro-2-phenylethyl)benzylamine (Lilly 08353)	2.7 × "
2-Benzyl-2-imidazoline (Priscol)	10^{-4}
Dihydroergotamine methanesulfonate	2 × "
2-(1-Piperidylmethyl)-1,4-benzodioxan (Benodaine, 933 F)	5 × "
Diethylaminoethanol	1.8 × 10^{-3}

* Mixture of equal parts of dihydro-ergocornine, -ergokryptine and -ergocrystine methanesulfonates. Molar concentration calculated on basis of mean molecular wt.

Salicylaldehyde oxidation is inhibited by NaCN (3.8 x 10^{-6} M). No inhibition was observed with iodoacetic acid, *p*-chloromercuribenzoic acid, ethylenediamine tetraacetate, ascorbic acid, sulfanilamide and two isonicotinic acid derivatives which inhibit monoaminoxidase (isonicotinylhydrazine) and diaminoxidase (1-isonicotinyl-2-isopropylhydrazine)(2). The reaction is not influenced by the various B vitamins, xanthine and its derivatives (adenine, guanine, allantoin, theobromine) and the following alkaloids: atropine, eserine, morphine, pilocarpine, strychnine. Other inactive compounds were: barbital, phenobarbital, thiourea, procaine, percaine, tetraethylammonium bromide, coramine, hesperidin and polyoxyethylene sorbitan monolaureate (Tween 20).

In view of the fact that both adrenergic amines and agents blocking adrenergic action inhibited aldehyde oxidation, it seemed important to determine whether these 2 groups of substances had the same type of inhibitory action. When reaction velocity was plotted against enzyme concentration the slope of the line obtained was not influenced by the concentration of inhibitor in the case of Dibenamine. With epinephrine, however, this slope was found to be a function of inhibitor concentration. According to Ackermann and Potter(3), this means that Dibenamine combines irreversibly with the enzyme while epinephrine causes only a reversible inactivation. By using other tests based on the influence of substrate concentration on reaction velocity (4), it was possible to show that inhibition by epinephrine and also by acetaldehyde is of a competitive nature.

Discussion. Three enzymes have been described which can oxidize aldehydes in the liver. The first, aldehyde mutase, converts aldehydes into an equimolecular mixture of the corresponding acid and alcohol(5). The enzyme dealt with in the present study does not produce any salicylalcohol and differs from aldehyde mutase by the fact that it is not inhibited by iodoacetic acid(5). Xanthine oxidase which also oxidizes aldehydes is different from the enzyme considered in this paper in that it is inhibited by ascorbic acid (6). Moreover, liver extract, as prepared in the present study, does not oxidize xanthine and xanthine oxidase prepared from milk is not inhibited by Dibenamine.

Liver aldehyde oxidase, as described by Gordon *et al.*(7) and studied by Knox(8) is very similar to the enzyme described above. Its relative affinity for salicylaldehyde and crotonaldehyde is compatible with the results just mentioned. Some minor differences are probably due to the differences in technics used. The results show that there are present in the liver extracts at least 2 enzymes capable of oxidizing salicylaldehyde. Only one of these is inhibited by the compounds investigated.

The most important question raised by the results of the present work concerns the possible role of aldehyde oxidase in the pharmacological actions of its inhibitors. It has been assumed that adrenergic blocking agents acted by inhibiting some hypothetical mechanism essential for the action of adrenergic substances(9). It was seen that aldehyde oxidation is inhibited by both adrenergic and adrenolytic agents. The fact that inhibition by adrenergic amines is reversible and competitive suggests that the aldehydes formed from them by the action of monoaminoxidase have a greater affinity for aldehyde oxidase than salicylaldehyde. This possibility, however, is difficult to investigate because of the extreme instability of these aldehydes.

There is a fairly good parallelism between adrenergic blocking activity and inhibition of aldehyde oxidase, but it is weakened by the failure of the potent adrenolytic drug, yohimbine, to inhibit the enzyme. The latter fact may be due to some metabolic change that yohimbine has to undergo before exerting its blocking action.

Inhibition caused by some antihistamine drugs leaves open the possibility of aldehyde oxidase also taking part in their pharmacological action. This, however, can perhaps also be explained by the well known overlap between adrenolytic and histaminolytic actions. It was shown that the action of aldehyde oxidase is reversible(8); under anaerobic conditions, the enzyme reduces acids to aldehydes. The possibility is now being studied that this action is somehow related to the pharmacological properties of the inhibitors. This and other investigations may show whether the facts reported in this paper are coincidental or are associated with the mechanism by which adrenergic compounds exert their action.

Summary. A method based on the ultraviolet spectrum of salicylaldehyde and sali-

cylic acid is presented for the study of liver aldehyde oxidase. By the use of this method it was observed that aldehyde oxidase is inhibited by epinephrine and other adrenergic amines as well as by adrenergic blocking agents. It was observed that inhibition by the first group of substances is reversible and competitive while inhibition by adrenolytic drugs is irreversible and non-competitive.

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Ionization Constants of Basic Dyes in Relation to Gastric Secretion.* (20286)

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Since Ingraham and Visscher(1) have shown that basic dyes are secreted by the stomach, a study was undertaken to see whether or not there was a relation between the ionization constants of the dyes and their ability to be secreted by the stomach. Ray and Jung(2) have determined the ionization constants of basic dyes from titration data in unbuffered 50% alcoholic solutions, and attempted to correlate them with their tendency to be secreted by the stomach. However, Ray and Peters(3) noticed certain discrepancies in their data. In the hope of finding an explanation for these discrepancies, Woislawski (4) redetermined the ionization constants of the dyes, which Ray and Jung have used, by titration in aqueous medium. He also noticed discrepancies between his values and those found by Ray and Jung.

The present study was undertaken because it was hoped that the determination of the ionization constants of the dyes by a different method(5), namely, from spectrophotometric data in buffered 50% alcoholic solutions, might shed some light on these discrepancies.

Method. A stock solution was prepared by dissolving 1 x 10^{-4} M of the dyes in 95% alcohol. To 10 ml of the stock solution in a 100 ml volumetric flask was at first added either 50 ml N HCl, N/10 HCl, phthalate buffer pH 3.0, acetate buffer pH 5.0, phosphate buffer pH 6.7, borax-HCl buffer pH 8.2, N/10 NaOH or N NaOH, which was then diluted to the mark with 95% alcohol. The final concentration of the dyes was thus 1 x 10^{-5} M. In both the buffered and unbuffered solutions the pH shifted toward the alkaline region upon dilution with alcohol. The absorption spectra were measured using a Beckman Model DU spectrophotometer. Spectra were measured from 400-700 mμ at 24 ± C. For the determination of pH a Beckman pH meter (model G) with glass electrode 1190T and calomel electrode 1170 was used. When the pH rose above 10 a Beckman glass electrode 1190E was used. Because the pKa cannot be converted to pK_B with any degree of accuracy in 50% alcoholic solutions, only pKa values are given in this paper. The pKa of the dyes was determined by the formula of Flexser *et al.*(6), $pKa = pH_m + \log E_B\text{-}E_m/$

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† Deceased April 16, 1953. Paper prepared for publication by F. E. Ray.

TABLE I. Comparative Values of Basic Ionization Constants Obtained by Different Methods.

Compound	pKa (buffered 50% alcohol)	pKa* (water)	pKa† (unbuffered 50% alcohol)	Buffer*	Stomach‡
				Benzene	Blood
Nile blue A	1.6	3.1	2.4	§	.8
Bismarck brown Y	4.5	4.8	5.0	§	26.5
Rhodamine B	4.6	3.7	3.2	¶	6.1
Acridine red	5.1	8.1	3.1	¶	8.4
Chrysoidin Y	5.5	5.0	5.3	§	75.0
Neutral red	6.8	6.7	6.5	¶	26.4
Pyronine B	7.1	6.9	7.7	1.96	5.7
Neutral violet	7.3	6.6	3.2	.68	27.2
Methylene green	9.7	4.1	3.2	2.50	13.6
Brilliant cresyl blue	9.9	4.6	3.2	2.70	11.3
Toluidine blue O	10.8	7.9	7.5	2.90	36.4
Thionin	11.2	6.8	6.9	11.60	36.8
Safranin O			6.4	5.60	27.3
Methylene blue			3.8	‖	15.6

* Ref. 7. † Ref. 4. ‡ Ref. 1. § 100% in benzene. ‖ 100% in buffer.
¶ Colorless.

E_m-E_{BH+} where E_B is the optical density of the uncharged molecule of the dye, E_{BH+} that of the charged ion, and E_m represents the density of the mixture of the 2 species; pH_m is the pH of the solution containing a mixture of the 2 species. For very strong bases it was not possible to determine the pKa because the dyes changed color near or above pH 13 which is beyond the limit of the pH meter. In those regions where the concentrations of the uncharged molecule and charged ion were approximately equal, additional spectra for solutions which differed only by a few tenths of a pH unit in either direction were determined.

Results and discussion. In Table I, column 2, the pKa values of the dyes are arranged in the order of increasing basicity in buffered 50% alcoholic solutions as determined from spectrophotometric data. In column 3 are listed the pKa values as determined from titration data in aqueous medium by Woislawski(4). In column 4 are listed the pKa values as determined by Ray and Jung(2) from titration data in unbuffered 50% alcohol. In column 5 are listed the distribution ratios between buffer pH 6.73 and benzene as determined by Woislawski(4). In column 6 are listed the Stomach/Blood ratios as determined by Ingraham and Visscher(1). Fairly good agreement for the ionization constants is obtained by all methods for Nile Blue A, Bismarck Brown Y, Rhodamine B, Chrysoidin Y, Neutral Red and Pyronine B. With the exception of Acridine Red, these are all the weaker bases. The spectrophotometric value is, however, close to the average of the other two. Higher values were found for the other dyes by the spectrophotometric method. The greatest discrepancy is the position of Bismarck Brown Y. With a low pKa it was a high rate of secretion by the stomach. Pyronine B, on the other hand, has a rather high pKa but a low rate of secretion. It seems probable that other factors are important in secretion. The three xanthenes: Pyronine B, Rhodamine B and Acridine Red, are secreted by the stomach in low concentrations (C_s/C_B = 5.7-8.4); their pKas are all low. The 3 azines: Safranin O, Neutral Violet and Neutral Red are much better secreted, and approximately to the same extent (C_s/C_B = 26.4-27.3); this in spite of the fact that Safranin O seems to be a much stronger base. Of the 4 thiazines, Methylene Blue and Methylene Green are secreted to a lesser extent than Thionin and Toluidine Blue O. Methylene Blue is known to undergo hydrolytic cleavage. It is probable that the dialkylimino group more readily undergoes this reaction *in vivo* with the formation of less basic oxazones. This decrease in basicity may account for their lessened secretion by the stomach. Methylene Green differs from Methylene Blue by an ortho nitro group which should further facilitate the hydrolytic cleavage of a dimethylimino group. The two oxazines Brilliant Cresyl Blue and Nile Blue A differ greatly in basicity, the former being a much stronger base. This alone could explain the greater secretion of the former dye. Nile Blue A is also known to undergo hydrolytic cleavage with the formation of a less basic oxazone. The two azo dyes Chrysoidin Y and Bismarck Brown Y are secreted by the stomach to a much greater extent than one would expect from the pKa values. It is quite possible that some other property plays an equally important role in secretion. Both of these compounds form chelates. While reducing the basicity this molecular configuration may be favorable to gastric secretion. We notice that the spectrophotometric pKa values largely parallel the buffer/benzene distribution ratios, column 5. The slight discrepancy between Neutral Violet and Pyronine B may well be within the margin of the error. Neutral Violet has a slightly larger pKa value than Pyronine B, but a larger buffer/benzene ratio. A more serious discrepancy is that Safranin O appears to be more basic than Thionin from spectrophotometric data, whereas the distribution ratios point to the reverse order. This discrepancy may be due to the limitations of the spectrophotometric method for very strong bases because the meso phenyl group of Safranine O should reduce the basicity. The fact that the ionization constants of the basic dyes have different values in different media is not surprising. It is obvious that the ionization of a base will be repressed in a more basic medium (pancreas) and increased in a more acid medium (stomach). This would result, however, in the ionization constants maintaining the same relative order. The data show that this is not the case. Woislawski(7) has shown that the order of diffusion also changes in different media.

Although the ionization constant of a compound undoubtedly plays an important role in diffusion one should be careful not to extrapolate ionization constants as determined in water to biological media. It was not possible to correlate satisfactorily the secretion of dyes in a biological system with their ionization constants in aqueous or buffered 50% alcoholic solution.

Summary. The ionization constants of 14 basic dyes belonging to 5 chemical classes, azo, azines, xanthenes, oxazines and thiazines, each containing 2-4 compounds, were determined spectrophotometrically in buffered 50% alcoholic solutions. The dyes were those which Ingraham and Visscher have found to be secreted by the stomach. Fairly good agreement for the ionization constants by 3 different methods (spectrophotometric in buffered 50% alcohol, titration in water and in 50% unbuffered alcohol) is obtained for Nile Blue A, Bismarck Brown Y, Rhodamine B, Chrysoidin Y, Neutral Red and Pyronine B. With the exception of Acridine Red these are all the weaker bases. Higher values were found for the other dyes by the spectrophotometric method. The spectrophotometric pKa values paralleled the buffer/benzene ratios. The order of basicity varies in different media. It was not possible to correlate satisfactorily the secretion of dyes in a biological system with their ionization constants in aqueous or buffered 50% alcoholic solution.

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Effect of Dehydroascorbic Acid in Rabbits. (20287)

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Patterson(1) reported that intravenous injection of dehydroascorbic acid, dehydroisoascorbic acid and dehydroglucoascorbic acid in rats (70-100 mg/100 g) led to hyperglycemia and glycosuria like alloxan. Like alloxan, the diabetogenic action of these substances could be prevented by a prior injection of either cysteine or glutathione(2). Patterson (3) also observed cataract in rats a few weeks after the injection of dehydroascorbic acid and found a linear relationship between the appearance of mature cataract and the blood sugar level of the animal. Profuse lachrymation and salivation were noticed just after the injection of dehydroascorbic acid of which the latter could be prevented by the destruction of the chorda tympani nerve and both by the injection of atropine. The toxic action of dehydroascorbic acid was much reduced by artificial respiration(4). Merlini, however, could not produce diabetes in rats by intraperitoneal injection of dehydroascorbic acid although he could observe lachrymation and salivation (5,6). Scurvy in guinea pigs where there is hypofunction of the islands of Langerhans as evidenced by diminished insulin content(7,8) and degranulation of the beta cells(9) is associated with the presence of dehydroascorbic acid and a decrease in the glutathione content of the tissues, whereas there is no dehydroascorbic acid in the tissues of normal guinea pigs(10). The present investigation was undertaken to study the effect of intravenous administration of dehydroascorbic acid in rabbits.

Experimental. Healthy Himalayan rabbits of weights varying between 1100 and 2000 g were used. Dehydroascorbic acid was prepared according to the directions of Patterson(1) and injected into the marginal ear vein of rabbits (1 g/kg) in 2 doses at intervals of 10 minutes, the initial dose was 250 mg. Repeated intravenous injections were also given in some animals. Immediately after the injection severe salivation and lachrymation were observed. Some of the rabbits died after the first injection while others survived after they were given artificial respiration. Sugar and glutathione values of blood were estimated in samples of blood taken at various intervals after injection of dehydroascorbic acid. Animals which survived the first injection were placed in metabolism cages for the collection of urine under toluene. Blood sugar was estimated by the method of Hagedorn and Jensen(11) and blood glutathione by the method of Woodward and Fry(12). Urinary sugar was estimated by the Benedict reagent. The results are given in Tables I and II. Six rabbits were given an intravenous injection of 0.25 g dehydroascorbic acid, followed after 10 minutes by another injection of the drug (1.25 g/kg). Only 2 of the rabbits survived the second injection and in these rabbits oral glucose tolerance test was performed after feeding a 50% solution of glucose (1 g/kg) on different days after the injection of dehydroascorbic acid (Table III). Blood sugar values of rabbits increased after injection of dehydroascorbic acid. To find out whether this rise was due to secretion of epinephrine, the effect of injection of dihydroergotamine, which inhibits the epinephrine-induced hyperglycemia(13), prior to the injections of epinephrine and dehydroascorbic acid in the rabbit was

TABLE I. Effect of Intravenous Injection of Dehydroascorbic Acid (1 g/kg) on Blood Sugar and Blood Glutathione Values of Rabbits (mg/100 cc Blood).

Wt of rabbit, g		Before inj.	Hr after inj. 1	2	3	24
1600	Blood S*	129	201	221	221	121
	Blood G	42	24	38	31	21
1300	Blood S	98	139	139	98	129
	Blood G	39	22	34	—	36
1500	Blood S	127	197	157	127	121
	Blood G	43	30	29	29	30
1350	Blood S	110	178	157	146	115
	Blood G	29	24	21	15	22

* Blood S = Blood sugar; Blood G = Blood glutathione.

TABLE II. Effect of Two Intravenous Injections of Dehydroascorbic Acid (1 g/kg) Repeated after 24 Hr on Blood Sugar and Blood Glutathione Values of Rabbits (mg/100 cc Blood).

Wt of rabbit, g	Before inj.	24 hr after 1st inj.	24 hr after 2nd inj.
	Blood sugar		
2000	119	112	139
1100	96	128	107
1600	129	121	132
1300	98	129	140
1500	127	121	127
1350	110	115	Dead
1300	110	—	129
	Blood glutathione		
2000	33	31	31
1100	40	34	30
1600	43	21	37
1300	39	36	32
1500	43	30	—
1350	29	28	Dead
1300	37	34	32

TABLE III. Oral Glucose Tolerance Test (1 g Glucose/kg) in Rabbits Injected with Dehydroascorbic Acid (1.5 g/kg).

Days after inj. when test done	Initial blood sugar (mg %)	Blood sugar (mg %) Hr after feeding glucose ½	1	1½	2½
1	107	160	171	171	175
3	107	171	192	181	128
7	128	164	164	171	130
1	133	233	—	270	216
3	123	203	266	308	282
7	131	200	223	212	178
12	135	189	178	178	132

studied. 0.2 mg of dihydroergotamine methanesulfonate* was injected in a rabbit and the blood sugar values at different hours after the injection were estimated. In another rabbit 20 μg of epinephrine was injected intravenously and blood sugar was estimated in different samples of blood. In 2 rabbits, half an hour after the injection of 0.2 mg dihydroergotamine, 10 μg of epinephrine was injected and the same dose of epinephrine was repeated after 45 minutes of the injection of the first dose of epinephrine. Blood sugar values were estimated at different hours after the injection. In another rabbit, half an hour before the intravenous injection of dehydroascorbic acid (1 g/kg), 0.2 mg of dihydroergotamine was injected and the blood sugar changes were recorded. The results are given in Table IV. Rabbits in which the injection of dehydroascorbic acid (1 g/kg) were repeated daily for 3 days died after the third injection. Tissues of these animals were studied histologically. Tissues were fixed in Zenker-formol solution and paraffin sections were cut and stained with azan and Heidenhain's iron-hematoxylin stains.

Results. After the injection of dehydroascorbic acid there was a rise in the blood sugar level for a few hours and the values returned to normal within 24 hours. Along with the rise in the blood sugar level there was a fall in the glutathione values of blood which also became normal after 24 hours. Urine of the animals contained albumin but no sugar was present. Repeated injections of dehydroascorbic acid for 3 days produced death of all animals. Glucose tolerance test carried out in 2 of the rabbits which survived the injection of dehydroascorbic acid (1.5 g/kg) did not show persistent diabetic type of glucose tolerance curve. The rise in the blood sugar level after the injection of dehydroascorbic acid could be prevented by a prior injection of adrenergic blocking drug—dihydroergotamine methanesulfonate. Histological studies showed changes in the kidney and testis and no changes were observed in the pancreas, suprarenal, pituitary and liver. In the kidney hyaline degeneration of some of the glomerulus and proximate tubules were observed. The glomerular tuft was atrophied and the space between the glomerulus and the Bowman's capsule increased. No change in the collecting tubules was observed. In the testis peripheral degeneration of the interstitial cells was observed.

Discussion. After the injection of dehydroascorbic acid there was profuse salivation, lachrymation, dilatation of the pupil and respiratory failure which indicated the stimulation of both the sympathetic and parasympathetic nervous systems. The initial rise in the blood sugar level seemed to be due to the secretion of epinephrine because this rise could be prevented by the adrenergic blocking drug—dihydroergotamine. The slight reduction of the blood glutathione level might be due to the

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* Kindly supplied by Sandoz Ltd., Basle, Switzerland.

TABLE IV. Effect of Intravenous Injection of Different Substances on Blood Sugar of Rabbits.

		Blood sugar (mg/100 cc blood)					
		Hr after inj. of substances					
Wt, g	Treatment	0	½	1	1½	2	3
1500	Inj. with 0.2 mg dihydroergotamine	124	—	115	—	94	99
1500	Inj. with 20 μg epinephrine	135	175	221	240	—	—
1500	Inj. with 0.2 mg dihydroergotamine followed by inj. of 10 μg epinephrine ½ hr and 1¼ hr after inj. of dihydroergotamine	88	88	95	97	85	63
2000	Same as above	83	95	95	104	90	63
1100	Inj. with 0.2 mg dihydroergotamine followed ½ hr later by inj. of dehydroascorbic acid (1.1 g)	96	107	89	—	84	

oxidation of glutathione and reduction of dehydroascorbic acid to ascorbic acid in the body. Persistent hyperglycemia and glycosuria could not be observed in any one of the rabbits injected with dehydroascorbic acid. The rabbits also did not show persistent diabetic type of glucose tolerance curve. No histological changes could be observed in the pancreas. All these findings indicate that dehydroascorbic acid is not diabetogenic in rabbits.

Summary. Intravenous injections of dehydroascorbic acid in rabbits in the dose 1 g/kg or 1.5 g/kg could not produce either persistent hyperglycemia or persistent diabetic type of glucose tolerance curve. Rabbits so treated neither excreted sugar in the urine nor showed any histological changes in the pancreas, suprarenal and pituitary. All these findings indicated that unlike rats dehydroascorbic acid is not diabetogenic in rabbits.

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Unidentified Factors Capable of Reducing Stress in Iodinated Protein-Fed Rats.* (20288)

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Liver preparations have been shown capable of partially relieving the stress produced in animals by a considerable number of experimental conditions. These studies have been summarized recently by Ershoff(1). The deleterious effects resulting from the administration of thyroid-active materials may be controlled under certain circumstances by yeast(2) and by several semi-purified protein materials(3,4) as well as by liver fractions when fed in conjunction with diets consisting of highly refined ingredients. The use of an assay for vit. B_{12} activity employing hyperthyroid rats in this laboratory(5,6) directed our attention toward a study of the differences in response of rats to vit. B_{12} when receiving highly purified and crude diets containing iodinated protein or other thyroid stimulants. Several investigators have observed that vit. B_{12} reduces the toxic effects of thyroactive materials when administered to animals receiving diets containing such crude constituents as yellow corn meal, soybean meal, cottonseed meal or dextrin(7-9) while the vitamin produces only slight beneficial effects when the animals receive purified diets (10,11). It is also significant that relief from other stress circumstances is given by vit. B_{12} when crude(12,13) but not when highly purified diets are employed(14). Work reported earlier(15) has demonstrated that either corn or soybean meal added to an otherwise purified diet in place of appropriate amounts of protein and carbohydrate makes possible a very significant response to vit. B_{12}. Further studies directed toward elucidating the reasons for the dissimilar responses of hyperthyroid animals to the two types of diets indicated that differences in production of vit. B_{12} or vit. B_{12f} could not account for the observed variations(16,17).

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In experiments preliminary to the present work in which levels of iodinated casein as high as 0.5% were employed, two facts became evident: a) Survival time of either adult or young rats was a more accurate measure of protection against such toxic amounts of this stress agent than growth or maintenance of body weight; and b) Certain diets containing crude constituents, notably pork or beef and high levels of corn and soybean meal, were capable of increasing the survival time of the animals even in the presence of adequate amounts of vit. B_{12}. While not the most refined measurement, survival data have yielded useful information concerning the protection afforded by the various dietary regimens while other indices were being evaluated. Studies arising from these observations are reported herein.

Procedure. In the experiments to be described, male weanling albino rats of the Sprague-Dawley strain with a weight range of 40-50 g were divided into identical groups and housed in individual suspended, mesh-bottomed cages. Food and water were supplied *ad libitum* and weights of the animals were recorded weekly. The basal rations employed contained 20-24% casein,[†] 0.3% cystine, 4% salts IV(18), 5% corn oil, 0.15-0.5% iodinated casein,[‡] vitamins and sucrose to 100%. The water soluble vitamins were employed at 5 times the levels described previously(16) with the exception of riboflavin used here at 25 and vit. B_{12} at 0.1 mg/kg. Fat soluble vitamins were administered as 4 drops weekly of a 1:1 haliver oil-corn oil combination containing 1 mg menadione and 2.5 mg α-tocopherol per ml of mixed oil. Except in cases which will be noted individually, the protein and fat levels of the supplemented diets were kept constant with those of the basals by appropriate reduction in the casein and corn oil levels. The cystine content of the various diets was reduced in proportion to the reduction in the casein level. Although several investigators(3,10,11) studying protection against the toxic effects of iodinated protein or thyroid material have added supplements at the expense of the carbohydrate of the diet, in view of the reports of protection afforded by increased levels of protein(19) or fat(20,21), such a practice was deemed inappropriate until adequate studies on the effects of these nutrients had been conducted. The corn-soybean diet employed in the first study reported in Table I contained equal quantities of yellow corn meal and soybean meal replacing the entire amounts of casein and sucrose of the basal diet. Ground whole pork was included in the third ration in this experiment at the expense of the casein and sucrose in an amount sufficient to produce a 21% protein level (N x 6.25). The meat was prepared according to previously reported methods(22).

Results and discussion. The preliminary findings indicating a high level of protection by the corn-soybean and pork containing

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[†] Vitamin test casein, General Biochemicals, Chagrin Falls, Ohio.

[‡] Protamone, Cerophyl Laboratories, Kansas City, Mo.

TABLE I. Influence of a Variety of Natural Materials on Growth and Survival of Rats Receiving Toxic Levels of Iodinated Protein (6 Animals per Group).

Exp.	Diets employed	Avg growth per wk (3 wk)*	Avg survival (days)†
1.	0.4% protamone		
	20% casein basal	23	26.4 ± 4.1‡
	21% pork protein	32	38.0 ± 5.2(1)
	Corn-soybean	30	56.2 ± 3.4(4)
2.	0.5% protamone		
	Ether extract of pork	25	22.2 ± 2.1
	Alcohol " "	25*	20.5 ± 1.6
	Water " "	26*	19.8 ± 1.3
	Extracted residue	26	40.8 ± 6.9(2)
	Dried whole pork	31	43.0 ± 4.9(1)
	20% casein basal	24	20.5 ± 1.1
3.	0.5% protamone		
	45% ground whole wheat	22	18.2 ± 1.7
	45% cooked fresh eggs	22	14.3 ± 0.3
	45% haddock	22	23.0 ± 3.6
	33.3% milk powder	15	14.0 ± 1.2
	20% alfalfa leaf meal	24	39.7 ± 5.6(1)
	20% barley malt	19	17.7 ± 1.7
	20% crude casein	24	17.8 ± 2.2
	15% yeast	23	29.3 ± 7.0(1)
	Basal + 10% cellulose	20	15.8 ± 1.2
	20% casein basal	16	14.0 ± 0.6
4.	0.5% protamone		
	20% alfalfa leaf meal	28	42.8 ± 6.4(1)
	Corn meal-soybean meal (10%+10%)	27	19.5 ± 1.6
	20% corn meal	23	23.4 ± 0.7
	20% soybean meal	27	24.8 ± 2.8
	19.8% haddock (dried)	23	21.0 ± 1.2
	20% yeast	30	35.3 ± 2.6
	20% eggs (dried)	29	25.3 ± 1.8
	20% whole liver (dried)	30	31.7 ± 2.1
	5% liver concentrate§	25	25.7 ± 3.2
	19.3% pork (dried and defatted)	25	49.7 ± 4.7(1)
	19.2% mutton (dried and defatted)	29	45.5 ± 6.6
	20% casein basal	25*	18.6 ± 1.9

* 2 wk growth reported in cases where fewer than half of animals survived 3 wk.

† Experiments terminated at end of 60 days. For those animals surviving entire experiment, 60 days has been used in the calculations of average survival. No. in parentheses indicate No. of animals surviving for 60 day period.

‡ Stand. error of mean, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$, where d = deviation from mean and n = No. of observations.

§ NF Powder, Wilson Laboratories.

diets are substantiated by the results of exp. 1. The difference between the survival of the animals receiving the basal and the pork rations is highly significant ($p < .01$) while even more protection is afforded by the corn-soybean mixture. Later work has indicated that the fat level of the ration containing whole pork (15%) can account for only part of the protection given by this diet.

Since meat may be separated into several fractions by simple procedures, in the next experiment an attempt was made to remove the protective activity from the major bulk of pork muscle. For this study the pork was dried, extracted with ethyl ether, then extracted with 95% ethanol and finally extracted with water. As a positive control dried whole pork was fed at a protein level comparable with the casein basal, and the various fractions were fed at levels comparable to their content in the whole pork ration. In each case the nitrogen level was increased to that of the basal with additional casein. The data indicate that little if any of the protective activity was removed by the extraction procedures. This observation agrees with the work of Ershoff(10) and Graham *et al.*(3,23) in which the "antithyrotoxic" properties of liver and beef muscle have been found to remain in the extracted residue.

In the last two studies reported in Table I a variety of materials have been assayed for their ability to prolong the life of rats receiving large amounts of iodinated casein. The most active materials found, listed in approximate order of decreasing potency, are mammalian muscle, alfalfa leaf meal, yeast and whole liver. Although Graham *et al.*(3) found whole egg protein to be active in maintaining growth and survival in their assay, the two trials reported here indicate a rather low content of the anti-stress activity for this material. Both the eggs and the haddock were dried before incorporation into the rations for Exp. 4 since it seemed possible that their high moisture content in the previous experiment might make the protamone more available or more toxic to the animals. While the eggs increased somewhat in activity when dried, neither material can be considered to be highly protective. Whole wheat, milk powder, crude casein, corn or soybean meal, when they constitute only 20% of the diet, or liver concentrate, NF powder, are also poor sources of the protective factor(s). Cellulose added to the basal diet also gave insignificant protection, indicating that the bulk content of the crude diets does not account for the protective action.

To ascertain whether our survival assay could be correlated with the growth assays of other investigators, the experiment employing 0.15% protamone shown in Table II was initiated. With this relatively low level of protamone, the growth data correspond very closely to the degree of protection by pork and alfalfa as measured by the survival assays (Table I). Growth data have frequently not correlated closely with survival data in the experiments employing 0.4-0.5% protamone. Also shown in Table II are the weights of several organs for normal rats and for animals receiving both 0.5 and 0.15% iodinated protein. Under both conditions the thymus is atrophied in the basal groups but is maintained at almost the normal size in the protected animals. In these studies the spleens of the protected animals are larger than those of the unprotected animals, while the adrenals of the rats receiving 0.15% protamone are hypertrophied less in the groups receiving alfalfa and pork than in the basal group. The kidneys of all the groups receiving iodinated casein are enlarged but are consistently reduced in size by the protective substances. Contrary to the findings of Ershoff(10) and Meites(13), no significant reduction in size of the testes is observed in the rats receiving iodinated casein. Differences in diet and susceptibility of the animals may account for the discrepancies. The seminal vesicles are partially restored to normal in some of the protected groups.

According to the descriptions by Selye(24) of the manifestations of general stress, the thymuses, spleens, adrenals, kidneys, and seminal vesicles of the rats show protection by some or all of the active materials against the stress induced by iodinated casein administration. The presence of vit. B_{12} in our diets may prevent the hypertrophy of the spleens in unprotected hyperthyroid animals as reported by Graham, *et al.*(23). The prevention of such hypertrophy by vit. B_{12} observed by these workers supports such a pro-

TABLE II. Influence of Protective Materials on Growth and Organ Size of Young Rats.

Regimen* and exp. period	Surviving rats/Total No.	Avg gain of surviving rats	mg/100 g body wt					
			Thymus	Spleen	Adrenals	Kidneys	Seminal vesicles	Testes
No protamone—3 wk exp.								
Normal controls	10		303 ± 14	369 ± 15	18.7 ± 0.8	835 ± 10	56.9 ± 3.3	1196 ± 42
No protamone—5 wk exp.								
Casein basal	6/ 6	196 ± 9.2						
Basal + pork	6/ 6	209 ± 12.2						
.5% protamone—3 wk exp.								
Casein basal	8/11	62 ± 4.2	244 ± 22	333 ± 20	37.8 ± 2.8	1688 ± 29	21.3 ± 9.3	1096 ± 46
Basal + pork	12/12	77 ± 5.7	274 ± 20	455 ± 27	35.5 ± 1.7	1466 ± 33	34.4 ± 4.5	1252 ± 39
" + alfalfa	5/ 6	90 ± 4.1	322 ± 24	462 ± 10	37.2 ± 1.5	1397 ± 23	31.2 ± 5.1	1135 ± 77
" + yeast	6/ 6	88 ± 5.1	290 ± 20	519 ± 59	29.5 ± 2.6	1393 ± 43	21.9 ± 6.5	1289 ± 41
.15% protamone—4 wk exp.								
Casein basal	4/ 5	111 ± 6.3	214 ± 17	355 ± 16	38.3 ± 3.9	1.65 ± .34†		1.40 ± .09†
Basal + alfalfa	5/ 5	129 ± 8.4	300 ± 33	476 ± 64	29.9 ± 4.6	1.27 ± .08		1.25 ± .09
" + pork	5/ 5	136 ± 3.6	281 ± 14	496 ± 19	27.6 ± 3.7	1.38 ± .35		1.27 ± .07

* Basal diets in these experiments contained 24% casein. Supplements were added at 20%.

† Grams.

posal. In view of the results of these studies, it is suggested that the presence of anti-stress factors in crude diets makes possible the previously considered vit. B_{12} response in hyperthyroid or otherwise stressed animals while similar animals receiving purified diets are deficient in both vit. B_{12} and these uncharacterized factors and therefore cannot respond to vit. B_{12} alone.

Summary. 1. A variety of natural products have been examined by means of a rat-survival assay for their ability to relieve the stress induced by iodinated casein administration. Mammalian muscle and alfalfa have been found to be excellent sources of the protective material(s) being measured, with liver and yeast also containing significant amounts. 2. Materials most active in the survival assay have been demonstrated to reduce thymus and seminal vesicle atrophy and kidney enlargement. When low levels of iodinated protein are employed, the adrenals are also partially restored to normal by the protective substances. Under the conditions employed, the spleens are significantly larger in the protected than the unprotected animals and the testes are not reduced in size by the thyroprotein administration.

We are indebted to Merck and Co., Rahway, N. J., for some of the crystalline vitamins and to Abbott Laboratories, N. Chicago, Ill., for haliver oil.

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Intravascular Life Span of the Duck Red Blood Cell as Determined by Radioactive Selenium. (20289)

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Various methods have been employed to estimate the life span of the red blood cell in the human, as well as in experimental animals. The earlier methods in which differential agglutination technics were employed have been reviewed by Isaacs(1). Hawkins and Whipple(2) studied the life cycle of the red blood cell in the normal bile fistula dog by following the bile pigment output after blood destruction or blood withdrawal. Tagging the red blood cell with N^{15}(3), and with P^{32}(4), are the more recent methods used to estimate the life span of the red blood cell. Jope(5) reported that sulfhemoglobin in the blood of T.N.T. workers was found to persist and was still detectable after many weeks. The disappearance of sulfhemoglobin from the blood stream was related to the destruction of the red blood cell which contained it. Further, a linear disappearance of sulfhemoglobin was found which disproved random destruction. In these experiments on man it was estimated that the life span of the red blood cell was 116 days. McKerns and Denstedt(6) have used the disappearance of sulfhemoglobin from the blood as a measure of the intravascular life span of the erythrocyte in rabbits. Dod, Bierman, and Shimkin(7) applied this method in normal rabbits and mice, and in tumor-bearing mice. In a previous publication(8) it was postulated that *in vivo* selenohemoglobin, similar to sulfhemoglobin, could be formed. The fixation of selenium in red blood cells of the rabbit(9) and the dog(10) has been demonstrated, and is shown below in the duck.

In view of the likely possibilities of similar properties of sulfhemoglobin and selenohemoglobin, experiments were carried out to study the rate of destruction of duck red blood cells tagged with radioselenium. The disappearance of selenohemoglobin from the circulation of the duck was therefore studied in detail. It was estimated that the intravascular life span of the duck red blood cell was 11.7 days.

Procedure. Two adult white Pekin ducks weighing 2.0 and 2.5 kg were injected intraperitoneally with sodium selenate containing radioselenium. Each duck received 2.5×10^6 c.p.m. radioselenium (8.0 mg selenium) in 3 doses, the second and third doses being administered 16 and 22 hours respectively after the first dose. The ducks were bled from the jugular vein 2 hours after the last injection. The citrated blood of the 2 ducks was pooled, centrifuged, the plasma separated, and the red blood cells washed repeatedly with isotonic saline. The washed, packed red blood cells were stored in the refrigerator until used, then mixed with an approximately equal volume of isotonic saline. This constituted the stock mixture which was administered the same day to recipient ducks. Each of 10 young white Pekin ducks weighing 481-567 g was injected in a leg vein with 10 ml (6 ml packed red blood cells) of the stock mixture which contained red blood cells tagged with radioselenium. Each duck received a total of 1.74×10^3 c.p.m. of radioselenium. Two each of the recipient ducks were exsanguinated intracardially as completely as possible after one hour, one day, 3 days, 7 days, and 10 days. The citrated blood was centrifuged, plasma aspirated, and the R.B.C.'s washed repeatedly with isotonic saline. The volume of packed red blood cells for each duck was recorded. The washed red blood cells were then wet ashed with concentrated HNO_3 and 30% H_2O_2, and radioactivity measurements were made similar to those previously outlined(8,11). In a separate experiment crystalline hemin was isolated from the blood of 4 ducks that had been injected with varying amounts of sodium selenate containing radioselenium. The methods used have been described previously(10). It was observed that samples of crystalline hemin contained 72.9, 177.1, 1008.8, and 396.4 c.p.m. per mM.

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TABLE I. Disappearance of Injected Red Blood Cells Tagged with Radioselenium (Selenohemoglobin) from the Blood of Ducks.

Duck No.	Time after injection	Body wt, g	Observed activity, c.p.m./ml R.B.C.	Corrected activity,* c.p.m./ml R.B.C.
702	0	539		
	1 hr	—	73.2	
703	0	567		
	1	—	103.5	
			Avg 88.4	
704	0	539		
	1 day	580	89.8	97
708	0	481		
	1	530	64.5	71
			Avg 77.2	Avg 84
705	0	510		
	3 days	630	61.2	75.9
706	0	510		
	3	630	54.6	67.7
			Avg 57.9	Avg 71.8
709	0	539		
	7	907	18.2	30.6
711	0	510		
	7	794	18.3	28.6
			Avg 18.3	Avg 29.6
710	0	481		
	10	935	7.4	14.3
713	0	481		
	10	964	8.1	16.4
			Avg 7.8	Avg 15.4

* Corrected activities were obtained by multiplying observed activities by a correction factor which was obtained by dividing body wt at time T by body wt at time O (see text).

Results and discussion. In Table I are shown the activities of duck red blood cells at various time intervals after the intravenous injection of red blood cells tagged with radioselenium. The results are expressed as c.p.m. per ml of R.B.C.'s. It was necessary to correct (column 5) the observed activities in order to account for the dilution of the tagged cells produced by the increase with age of total blood volume. The corrected activities were calculated by multiplying the observed activities by a dilution factor which was obtained by dividing the body weight at time T by the weight at time O. Since hematocrits and percentages of body weight as blood remain essentially constant during the experimental period, the ratio of red blood cell volumes is proportional to the weights. It will be noted that the average activity value for 2 ducks at each time T shows a progressive decline with time.

The first blood samples taken at the one-hour interval (Ducks 702 and 703) represent well-mixed and maximum blood activity samples. If the average value 88.4 c.p.m. per ml R.B.C. for the first hour samples is taken as the maximum or initial blood activity level, the percent of original activity for the 1st, 3rd, 7th, and 10th days would be 95.1%, 81.3%, 33.5%, and 17.4%, respectively.

In Fig. 1 is shown a plot of R.B.C. activities (% of initial value) against time in days. The straight line was constructed on the basis of least squares of perpendiculars to the line, giving a line described by: $y = 100–8.5x$, where y = percentage of injected activity, and x = time in days. It will be noted that the line intersects the x axis at 11.7 days. This value of 11.7 days is interpreted to represent

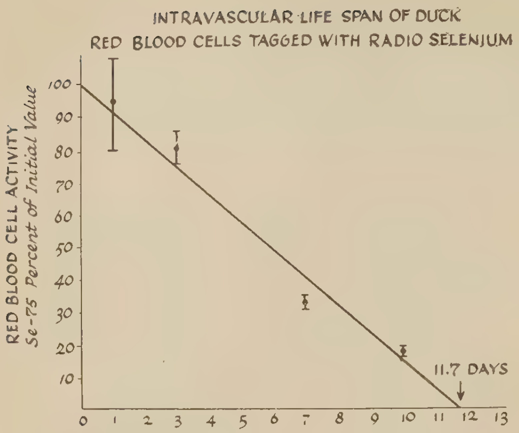

FIG. 1.

the intravascular life span of the duck erythrocytes under experimental conditions described here. The linear disappearance of selenium-tagged red blood cells in the duck indicates, as Jope(5) has shown in the case of sulfhemoglobin in man, that the red cell has a fixed life span. To further clarify the assumption that the x-intercept does in fact represent this mean erythrocyte life, it may be recalled that the donor cells were of all ages but that presumably the mean age of the donor erythrocyte is exactly mid-life. The point of disappearance of activity from the cell presumably represents the destruction of the youngest donor cells. If the Jope interpretation of straight line disappearance is correct, this x-intercept also represents the mean intravascular life of the erythrocyte. Further, the linear disappearance of selenium from the red cell fraction of blood is interpreted to be related to the destruction of red cells, and to show that this process does not take place at random. In correlating the relationship between the disappearance of selenohemoglobin (fixed selenium) and the destruction of the red cells, certain assumptions are made similar to those of Jope(5) on the disappearance of sulfhemoglobin from the blood. It is assumed that the intact red cell does not have the ability of exchanging fixed selenium, and that the organism has no way of removing it other than removal of the red cells which contain selenium. When the cells containing fixed selenium are broken down, the selenium is not incorporated into new red cells. In relating the destruction of the red cells containing selenium to that of normal cells it must be assumed further 1) that the selenohemoglobin formed is distributed initially at random among the red cells of all ages existing in the donor duck at that time, 2) that the formation of selenohemoglobin within the cell does not prolong or curtail its life span, and 3) that the polycythemia induced by donor cells does not materially accelerate destruction.

As far as we have been able to discover, this is the first report on the intravascular life span of the duck R.B.C. At first it appeared that the life span of the R.B.C. in the duck as determined here was very short. However, in another fowl, the hen, Hevesy and Ottesen(4) using P^{32}, and Shemin(12) using N^{15} glycine, have shown the life time of the red nucleated corpuscle to be 28 days, a relatively short time as compared with other species, for example man, which has been estimated to be 127 days(3).

Summary. Use has been made of the ability of selenium to be fixed in red blood cells to estimate the intravascular life span of the duck erythrocyte. Donor ducks were injected with sodium selenate containing radioselenium. These ducks were then bled and the red blood cells injected into 10 young ducks. Two each of the recipient ducks were bled at various time intervals up to 10 days. It was noted that there was a linear disappearance of selenium-tagged red blood cells from the circulation of the ducks. The value of 11.7 days was found to be the intravascular life span of the duck erythrocytes under the experimental conditions described here. In correlating the relationship between the disappearance of fixed erythrocyte selenium and the destruction of the red cell certain assumptions have been made which are discussed.

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Effects of Thyroxine, Thyroidectomy and Lowered Environmental Temperature on Incorporation of Deuterium into Cholesterol.* (20290)

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The effects of thyroid- and thiouracil-feeding on the rate of incorporation of heavy hydrogen isotopes into cholesterol were studied in the rat by Karp and Stetten(1), and by Byers and coworkers(2). However, since Fleischmann and coworkers demonstrated recently that α-naphthylthiourea influences cholesterol metabolism not only by way of the thyroid gland, but also by another mechanism, completely independent of the thyroid (3), the interpretation of the above mentioned results presents some difficulties in the case of the hypothyroid groups. It was decided, therefore, to produce a hypothyroid condition in rats by means of thyroidectomy, rather than using anti-thyroid drugs, and to study the rate of incorporation of deuterium into cholesterol in these animals, and, for comparison, in hyperthyroid rats. The results are reported in the present paper.

In the course of these experiments, another difficulty in interpretation arose, *i.e.,* whether the effects observed in the hyperthyroid group were the result of a direct and specific action of the thyroid hormone, or, whether they were solely the consequence of an increase in the metabolic rate in general. Attempts were made to obtain information on this question by means of using animals in which the metabolic rate was increased independently of the thyroid gland; this was accomplished by exposing thyroidectomized rats to a lower environmental temperature in accord with observations reported by Ring(4), and Sellers and You(5). The present report includes results of experiments in which the rate of incorporation of deuterium into cholesterol was compared in thyroidectomized rats kept at 2-5°C, and at 25-30°C.

Methods. Female weanling rats, USC strain, were fed *ad libitum* a diet low in cholesterol.[‡] Littermates were used as far as possible. In the first experiment which dealt with the effects of modifications of the thyroid activity, 3 groups of rats were used: One group was injected intraperitoneally with .05 mg thyroxine daily for a period of 3 weeks. Another group was thyroidectomized at an age of 4 weeks, and maintained for 2 weeks to deplete circulating thyroid hormone. Untreated animals of the same age served as

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[‡] Composition of diet: 33.8% oats; 33.7% whole wheat; 15.0% non-fat milk powder; 4.5% alfalfa; 2.0% yeast (strain G, Anheuser-Busch); 8.0% cottonseed oil; 2.0% fortified oil (containing 312 U.S.P. units vitamin A and 50 U.S.P. units vit. D per g); 0.5% calcium carbonate: 0.5% sodium chloride.

TABLE I. Effects of Thyroxine and Thyroidectomy on Incorporation of Deuterium into Cholesterol in the Rat.

Group	No. of rats	Mean deuterium content of body water, atom % excess	Mean deuterium content of tissue cholesterol as % of deuterium content of body water (atom % excess)* Liver	Intestine	Kidneys	Spleen
Thyroxine	15	1.51 ± .04	36.6 ± 1.2	42.2	24.4	17.5
Control	13	1.59 ± .04	27.7 ± 1.7	31.5	17.4	11.6
Thyroidectomized	5	1.79 ± .19	22.9 ± 1.0	—	—	—

* Liver samples were determined individually, samples of other organs were pooled.

controls. Deuterium oxide was administered to all groups during the last 4 days of the experimental period as follows: One initial injection of 25% v/v deuterium oxide was given intraperitoneally at a level of 3.9 ml per 100 g body weight, to bring the deuterium content of the body fluids to 1.5 atom % excess; for the remaining 4 days, deuterium oxide was furnished with the drinking water at a concentration of 2.5% (v/v). In the second experiment which concerned the effects of a lowering of the environmental temperature, 2 groups of female rats were thyroidectomized at 4 weeks of age and then maintained for 2 weeks without any treatment, as mentioned before. Deuterium oxide was then administered as described above, except that the period of administration was extended to 6 days. During this period, one of the groups was kept in the cold room, at a temperature of 2-5°C. The other group remained at room temperature which varied between 25 and 30°C. No thyroxine was applied to these animals. In all experiments, the animals were approximately 7 weeks old, when sacrificed. At that time, the livers, and, in some of the experiments, the intestines, kidneys, spleens and lungs were excised. In the case of the thyroidectomized animals, the region of the neck was carefully checked for completeness of the operation. All tissues were minced and extracted first with ethylalcohol, then with ethylether, using Soxhlet extractors. The solvents of the extracts were removed by evaporation, and the lipid residues were hydrolyzed by refluxing for 30 minutes in 50 ml acetone-alcohol 1:1, containing 6 ml of 33% w/v KOH. After neutralization, the total cholesterol content was determined in most of the samples in a small aliquot, using a modified Schoenheimer-Sperry procedure(6). The remaining cholesterol was precipitated as the digitonide, the precipitate washed with water and ethylether, and then dried in a vacuum desiccator. The cholesterol digitonide was combusted, and the collected water was reduced over zinc at about 400°C to a gas mixture containing hydrogen and deuterium as reported recently from this laboratory(7). The deuterium content of the gas mixture was determined using a Nier-type isotope ratio mass spectrometer (Consolidated Engineering Corp.). The body water samples, obtained from the carcasses by vacuum distillation, were purified by distillation over alkaline potassium permanganate. The deuterium content of these samples was measured as described above.

Results and discussion. The rats treated with thyroxine exhibited typical symptoms of hyperthyroidism such as an increased physical activity, tachycardia, an increased respiratory rate, and an increased food and water intake and fecal excretion; the thyroxine level was not high enough, however, to influence significantly the body weight gain. The thyroidectomized rats showed somewhat less pronounced changes in the opposite direction. The total cholesterol content of the organs examined was not significantly altered in the experimental groups, in agreement with observations by Karp and Stetten(1).

The results of the first experiment which concerned the effects of thyroxine and thyroidectomy on the incorporation of deuterium into cholesterol, are summarized in Table I. The data show that the deuterium content of the liver cholesterol was significantly higher in the hyperthyroid group, and significantly lower in the thyroidectomized animals, than in the controls. The same trend was observed for the intestine, kidneys and spleen. The values for the lungs showed practically no difference (not included in Table I). Computations of standard errors were not possible in the case of the latter organs, as the determinations were made on pooled samples. Since the deuterium content of the body fluids was practically the same in the 3 groups, within the limits of error of the procedure, the data were interpreted to indicate that the thyroid hormone stimulated the incorporation of deuterium into cholesterol in the liver, intestine, kidneys and spleen. These findings are essentially in agreement with observations made by Karp and Stetten on liver and carcass cholesterol(1), and by Byers and co-workers on cholesterol extracted from pooled viscera(2). These authors used different technics, however, to alter the thyroid activities of their experimental animals, *i.e.*, feeding of desiccated thyroid and thiouracil, respectively. As mentioned above, difficulties in interpretation arise, if data concerning cholesterol metabolism are obtained from rats treated with anti-thyroid drugs, since Fleischmann and co-workers showed that α-naphthylthiourea influences the metabolism of the sterol even in the absence of the thyroid gland(3). In the present work, these difficulties were avoided, since no sulfa drugs were used, and the observations made on the hypothyroid group can be related unequivocally to a thyroid deficiency.

A further difference in experimental conditions concerns the food and water intake of the experimental animals. In the experiments presented above, the rats had free access to food and water during the entire experimental period. Byers and coworkers, on the other hand, observed the incorporation of tritium into cholesterol under fasting conditions (both food and water were withheld).

The question, whether these results can be interpreted to indicate that thyroid hormone stimulates cholesterol synthesis or turnover, cannot be answered unequivocally until some other possibilities are ruled out. It is not known, *e.g.*, whether a hyperthyroid condition makes possible an exchange reaction between carbon-bound hydrogen and deuterium,§ or, whether thyroid causes primarily an increase in the deuterium content of smaller molecules which serve as precursors of cholesterol(1). However, Byers and coworkers concluded that thyroid stimulated the rate of cholesterol synthesis, not only on the basis of the reported observations on the incorporation of tritium into cholesterol(2), but also, because additional evidence along these lines was obtained from experiments on the relation of thyroid to biliary cholesterol excretion(9). Thus, the results presented above, when considered in conjunction with these reports, might be interpreted to indicate that thyroid stimulated the rate of synthesis or turnover of cholesterol.

Another difficulty in interpretation concerns the nature of the causative mechanism responsible for the observed changes in the rate of deuterium incorporation, *i.e.*, the question, whether the effects found were the result of a specific and direct action of the thyroid hormone, or whether they were solely the consequence of an increase in the metabolic rate in general. In order to obtain some information on this problem, it was decided to alter the metabolic rate independently of the thyroid gland, and to study the effects of this alteration on the incorporation of deuterium into cholesterol. Ring(4), and Sellers and You(5) have demonstrated that the metabolic rate is significantly increased, when an animal is exposed to a lower environmental temperature, even in the absence of the thyroid gland. Therefore, the incorporation of deuterium into cholesterol was compared, in the following experiment, in thyroidectomized rats exposed to an environmental temperature of about 2-5°C, and in those kept at about 25-30°C. The results summarized in Table II indicate that,

TABLE II. Effect of Environmental Temperature on Incorporation of Deuterium into Liver Cholesterol in Thyroidectomized Rats.

Environmental temp., °C	No. of rats	Mean deuterium content of body water, atom % excess	Mean deuterium content of liver cholesterol as % of deuterium content of body water (atom % excess)
2– 5	9	1.52 ± .14	29.3 ± 3.1
25–30	6	1.48 ± .12	29.1 ± 1.9

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§ According to Schoenheimer and Rittenberg, carbon-bound hydrogen is "stably bound", excepting those hydrogen atoms which are attached to a carbon atom adjacent to a keto group(8).

in the absence of the thyroid, a lowering of the environmental temperature did not modify the deuterium content of the liver cholesterol under the conditions of this experiment. These data were interpreted to indicate that an increase in the metabolic rate alone did not influence the incorporation of deuterium into cholesterol in the liver. It was concluded, therefore, that the phenomenon observed in the hyperthyroid rats as reported above was similarly not mediated primarily by an increase in the metabolic rate, but that it was very likely a direct consequence of the increased thyroid activity.

Summary. 1. The incorporation of deuterium into cholesterol was studied in rats injected with thyroxine, in normal rats and in thyroidectomized rats. 2. It was observed that thyroxine stimulated, and thyroidectomy depressed, the incorporation of deuterium into cholesterol in the liver, intestine, kidneys and spleen, but not in the lungs. 3. In thyroidectomized rats, a lowering of the environmental temperature by approximately 20-28°C did not alter the rate of incorporation of deuterium into cholesterol in the liver.

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Response of Dogs to Intravenous Administration of Polyvinylpyrrolidone and Its Monomer.* (20291)

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Hecht and Weese(1) first reported the effective use of polyvinylpyrrolidone (PVP), a fractionated polymer of N-vinylpyrrolidone synthesized from acetylene and formaldehyde, as a plasma volume expander in clinical shock. At that time they also showed that the dog exhibits a sensitivity to PVP; this observation has been confirmed by several other investigators(2,3,4). Untoward reactions to PVP, used as a therapeutic agent in shock, have not been encountered either in man or other species of laboratory animals(1,4-8). During a survey of the more recent colloidal solutions recommended for use in clinical shock, the authors found that PVP was inferior to certain other colloids tested in dogs subjected to hemorrhagic shock; undoubtedly the peculiar canine reaction, in which hypotension and hemoconcentration are the primary manifestations, contributed to these results. Attempts to render the dog less sensitive to infusions of PVP by prior small injections(1,2) failed in our hands.

It was considered of interest to attempt to delineate the cause of the "canine reaction". In view of the use of cortisone in certain hypersensitive conditions and its positive effect in the rat after injection of dextran(9, 10) and human globin(10), this steroid was used to determine its effects on the reaction of the dog to PVP. Thus, the purpose of this investigation was two-fold: 1) to trace down the component of PVP solutions responsible

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TABLE I. Blood Pressure and Hematocrit Data after Injection of PVP (3 cc/kg) of Various Molecular Sizes and Its Monomer.

		Avg values after inj.—% of init.				
		Blood pressure			Hematocrit	
Treatment	No. of cases	1-3 min.	5 min.	30 min.	5 min.	30 min.
PVP (clinical)	6	41	45	52	117	138
" (K-21)	3	33	41	41	122	129
" (K-62)	6	108	96	99	98	103
Monomer (170 ×)						
45 sec. inj.	3	61	94	99	108	108
Rapid inj.	4	52	79	90	105	102
Cortisone + PVP	5	58	75	89	106	104

for the untoward reaction, and 2) to determine to what extent the adverse hemodynamic responses detract from the colloid oncotic properties of PVP in "shocked" dogs.

Procedure and methods. Mongrel dogs were anesthetized with pentobarbital sodium and a femoral artery was cannulated when needed for recording blood pressure and for bleeding. Some unanesthetized dogs were also used in which case blood pressure was determined by arterial puncture. Blood samples were collected in heparinized tubes at various intervals and hematocrits were determined in Wintrobe tubes centrifuged for 30 minutes at 3200 rpm, radius 14 cm (to end of tube). For the experiments on bled dogs, 2 hemorrhage procedures previously reported(11) were employed: Procedure I—acute hemorrhage to a blood pressure end point of 20 mm Hg followed by immediate infusion of a volume of replacement fluid equal to the volume of hemorrhage; Procedure II—acute hemorrhage to 30 mm Hg with one hour of hypotension at this pressure intervening before infusion of an equal volume of substitute.

Results. I. *Reaction of intact dogs to injections of PVP and vinylpyrrolidone* (monomer of PVP). Table I lists the various materials used and shows their effect on blood pressure and hematocrit at intervals up to 30 minutes. Unanesthetized and anaesthetized animals did not differ significantly in their blood pressure and hematocrit responses to PVP; consequently, anaesthetized dogs were used primarily.

a) *Clinical PVP.*[†] Animals were variable in their reaction to very small injections. Of 2 dogs injected with as little as 0.03 cc/kg, one showed an immediate fall in blood pressure from 135 to 55 mm Hg and returned to normal within 10 minutes, while the other was not affected. Doses of 0.37 cc/kg and 0.5 cc/kg failed to produce severe reactions in all dogs tested; an unanesthetized animal receiving the larger dose exhibited marked flushing of the skin, labored respiration, cyanotic tongue, feeble pulse, unsteady gait, and shaking and scratching of the head, whereas a second dog showed only mild symptoms. Consistently severe reactions occurred in all 6 dogs receiving 3 cc/kg injected in 45 seconds (equivalent to a rate of 4 cc/kg/min). Even by 90 minutes after this dose, the average blood pressure was only 67% of initial and the average hematocrit remained elevated to 134% of the initial value. After one to 5 hours in those animals receiving 3 cc/kg, the blood pressure rose slowly and was maintained at a lower level than the initial. If a second injection was now administered, then the severity of the accompanying reaction was considerably reduced, and in some instances, no apparent effect was noted. Twenty-four hours after the first injection, however, a third injection of the same magnitude elicited a marked reaction.

b) *K-21 PVP, K-62 PVP and monomer of PVP.*[‡] Since the clinical preparation of PVP, as well as other colloidal solutions used as plasma volume expanders, consists of a material with a wide range of molecular sizes, it was considered pertinent to use "narrow cuts" at both ends of the range to ascertain the relative effects of small and large molecules of the polymer. Also present in the clinical PVP solution is approximately 17.5 mg % vinylpyrrolidone(13); this material was injected intravenously in amounts varying from 1 to 170 times that present in the clinical solution, both undiluted and diluted in saline to a volume of 3 cc/kg. Data presented in Table I show that K-21 PVP produced practically the same profound reaction as the clinical preparation, whereas the K-62 material caused only insignificant effects. With the smaller molecular weight material even at 90 minutes after injection, the blood pressure remained depressed at 59% of initial and hematocrit was elevated to 125% of initial; with the larger, there was a slight hypertensive effect immediately after injection followed by a slight decline in blood pressure and then a return to normal levels (slight flushing did occur in an occasional animal with K-62 PVP). Quantities of pure liquid vinylpyrrolidone up to 170 times that found in clinical PVP, diluted in saline and injected in volumes of 3 cc/kg, produced no significant changes. Injected undiluted in the largest amount, the monomer caused transitory hypotension, which was more protracted with the rapid rate of injection (Table I).

[†] 3.5% Solution, Schering Corp. Lot GA-PVP-111; K-value 33.5, Weight average molecular weight 81000(12). Allocated by the National Research Council.

[‡] Powdered PVP (K-21 and K-62) and pure liquid vinylpyrrolidone were supplied through the courtesy of Dr. J. C. Howard, Jr., Schenley Laboratories. K-21 and K-62 PVP were compounded in sterile 0.9% saline as 3.5% solutions; the reported average weight molecular weights are 20500 and 560000 respectively(14).

c) *Prophylactic treatment with cortisone before injection of clinical PVP.* Five dogs were injected with cortisone intramuscularly for five days prior to an intravenous injection of 3 cc/kg of clinical PVP. One animal received 5 mg per day, one 10 mg, and three 50 mg per day. The steroid was injected once daily, half of the dose in each hind quarter. On the morning of the sixth day these animals were injected with 3 cc/kg of clinical PVP. Prophylactic treatment with cortisone did not completely nullify the "canine reaction" to PVP, but did decrease its severity and duration. By 30 minutes after the PVP injection (Table I), average blood pressure and hematocrit values for the cortisone-treated groups were approaching the initial values, whereas by 90 minutes the average hematocrit for the PVP group not receiving cortisone remained significantly elevated (134% of initial) and hypotension still persisted (B.P.: 67% of initial). Among the cortisone-treated animals only the one receiving the smallest dose exhibited marked flushing of the skin, 2 showed mild flushing, and 2 showed no flushing.

TABLE II. Blood Pressure Response to Infusions of Clinical PVP (13 Dogs).

PVP, cc/kg	Blood pressure—mm Hg			
		Min. after inf.		
	Init.	2–3	30	90
	Intact—4 cc/kg/min.			
40	90	40	30	55
40	140	65	120	130
40	100	60	65	85
	Bled—4 cc/kg/min.			
42.5	115	30	35	45
54.5	145	Died during infusion		
	Bled—2 cc/kg/min.			
50	155	60	95	90
28	150	45	60	90
35	160	55	80	100
43.5	130	60	95	110
37.5	120	40	75	100
	"Primed"—bled—4 cc/kg/min.			
24.3	95	50	50	75
40.4	90	75	95	110
21.1	80	40	40	80

II. *Response of intact and bled dogs to infusions of clinical PVP.* a. Effect of rate of infusion. The pertinent blood pressure data are presented in Table II. Three intact anaesthetized dogs were infused with 40 cc/kg at the rate of 4 cc/kg/min; no deaths occurred, but the typical reaction ensued (the magnitude of the infusion masked the hemoconcentrating effects produced by small injections). Two dogs were subjected to a controlled hemorrhage to a blood pressure end point of 20 mm Hg and were immediately infused at 4 cc/kg/min with a volume of PVP equal to the volume of blood removed; the blood pressure response of one of these dogs was extremely sluggish (39% of initial 90 minutes after infusion), and the other died before the end of infusion. No fatalities resulted in bled dogs infused at the rate of 2 cc/kg/min and blood pressure increased significantly over the immediate post-infusion

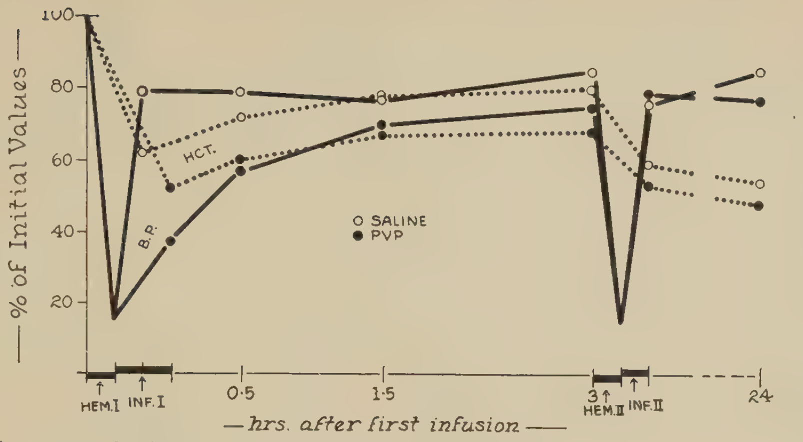

FIG. 1. Avg curves for blood pressure (solid lines) and hematocrit (dotted lines) for bled dogs on Procedure I.

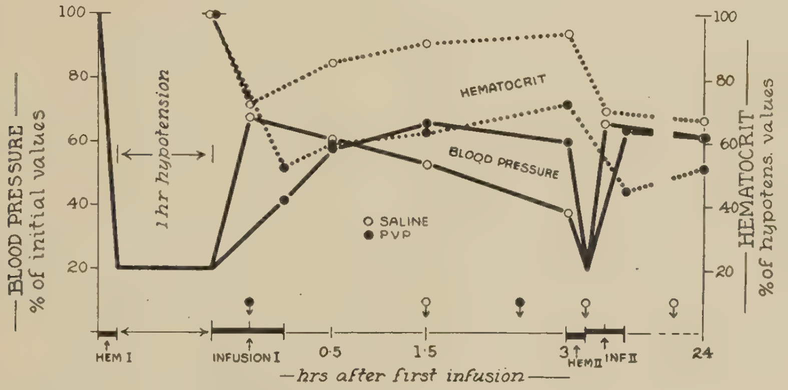

FIG. 2. Avg curves for blood pressure (solid lines) and hematocrit (dotted lines) for surviving bled dogs on Procedure II. Symbols with arrows represent time of death for each animal on indicated therapy.

levels. The bled dog could indeed tolerate the faster rate, if hemorrhage and infusion were performed two to three hours after a second (or third) small injection had been administered, *i.e.,* after the blood pressure had reached a plateau (*vide supra*). In view of the existing hypotension, however, it is highly questionable if such animals could be considered as fully recovered from the effect of the "priming" injections.

b) Evaluation as a Plasma Expander in the Bled Dog. Ten dogs were subjected to a controlled hemorrhage in accordance with Procedure I, infused immediately with PVP and 5 with physiological saline. Nine dogs were bled in accordance with Procedure II; 4 being infused with PVP after the delay of one hour and 5 with saline. Fig. 1 and 2 show average mean blood pressure and average hematocrit responses for the 2 procedures. In each instance the infusion rate for PVP was one-half that for saline—2 cc/kg/min in Procedure I, and 1 cc/kg/min in Procedure II for the initial infusion. The figures show clearly the delay in blood pressure response to PVP infusions. They also demonstrate that the PVP infusions caused a greater hemodilution as measured by hematocrit change than did saline, particularly in the more severely shocked animals. All animals survived on Procedure I, while several animals in each group succumbed before 24 hours on Procedure II (indicated by symbols with arrows in Fig. 2). To estimate the relative overall effectiveness of various infusion fluids, the ratio of subsequent bleeding volumes to the initial, termed the Bleeding Volume Index (BVI), has been employed. For Procedure I at 3 hours after infusion, the BVI's for PVP and saline were 64 and 54 respectively; for Procedure II, 23 and 12 respectively.

Comments. The foregoing data indicate that the smaller molecular weight portions of PVP produce the prolonged hypotensive effect observed in intact and bled dogs. Since amounts of vinylpyrrolidone equivalent to those encountered in clinical PVP produce insignificant changes, and only in excessive amounts does a transitory effect ensue, this substance may be ruled out as the causative agent of the "canine reaction." Whether the smaller molecules themselves act as a vasodilator component or whether they elicit the release of a vasodilator substance into the circulation remains for further investigation. In view of its hemoconcentrating effect and the partial benefits of cortisone, the possible influence of this material on capillary permeability is also indicated for additional study.

Polyvinylpyrrolidone is not recommended as a suitable therapeutic agent for shocked dogs. Although apparently superior to crystalloidal solutions in certain respects(15), PVP has not proven to be as effective in the dog as dextran, oxypolygelatin, or modified fluid gelatin under the same experimental conditions(16).

Summary. 1. Due to a species sensitivity, whose nature is as yet unknown, PVP cannot be considered as a suitable colloid replacement fluid for the treatment of "shocked" dogs. 2. Intact dogs were variable in their response to small intravenous doses, 0.03 to 0.5 cc/kg of clinical PVP, but reacted severely to intravenous injections of 3 cc/kg exhibiting hypotension, hemoconcentration, and flushing of the skin of several hours' duration. 3. A small molecular weight fraction of PVP (K-21) produced severe reactions, while with a larger molecular weight fraction (K-62) only insignificant changes occurred. 4. Monomeric vinylpyrrolidone, in quantities far in excess of those found in clinical PVP solutions, caused only transitory hypotension when injected undiluted. 5. Cortisone, used prophylactically over a period of 5 days prior to an injection of 3 cc/kg of PVP, decreased the severity and duration of the "canine reaction." 6. Bled dogs did not tolerate well an infusion rate of 4 cc/kg/min; no fatalities resulted from infusions at half this rate.

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Metabolic Effects of Growth Hormone in Fasting, Phlorhizinized and Adrenalectomized Rats. (20292)

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In the rat, anterior pituitary growth hormone has been clearly shown to promote nitrogen storage(1) and to diminish the proportion of carcass fat(2). In accord with this is the report(3) that in the fasted normal rat there is a lowering of the blood sugar level, decreased urinary nitrogen excretion and increase in ketone body production after administration of anterior pituitary preparations rich in growth factor. The relationship of these phenomena to the adrenal cortex is, however, not clear. In this same study(3) there is also reported a decrease in endogenous protein catabolism and increased ketonuria after saline A.P.E. in the adrenalectomized rat. Several other authors(4-7) have reported that the adrenal gland is not necessary for the ketogenic response to growth hormone. On the other hand, it has been reported(8-10) that the ketosis caused by anterior pituitary growth preparations is diminished or completely inhibited in the absence of the adrenals.

In 1940 Gaebler and Zimmerman(11) reported on the effect of a crude anterior pituitary extract on the metabolism of phlorhizinized fed dogs. They reported a diminution of glucose and nitrogen output which was not at the expense of increased fat oxidation. Since the metabolic action of growth hormone in the adult dog differs from that in the rat, a study was undertaken of the effect of purified growth hormone on the metabolism of fasted phlorhizinized rats. In addition, the relationship of the adrenal cortex to the effects of growth hormone both in the unphlorhizinized and phlorhizinized animal was studied.

Material and methods. Young mature female rats of the inbred Wistar strain, weighing from 150 to 225 g, were used. Bilateral adrenalectomy was performed in a single stage through a dorsal cutaneous incision under ether anesthesia. Following the operation the animals were fed Purina Laboratory Chow for 2 days. On the 3rd post-operative day food was removed and on the 4th day the animals were placed in metabolism cages and 50 mg/100 g body weight of phloridzin glucoside as 10% solution in propylene glycol was injected subcutaneously(12). 0.5 mg/100 g body weight of growth hormone* was administered intraperitoneally on each of 2 days prior to and on the day of urine collection. Throughout the course of the experiments all animals received 1% saline rather than tap water. Twenty-four hour urine specimens were collected in clean vessels and preserved with a crystal of thymol and one g of citric acid. Washings from the funnels were added to the day's collection. Urine glucose was determined by the Nelson Modification of the Folin Wu Micro-Method(13). Urinary NPN was determined after deproteinization by the Micro Kjeldahl procedure(14). Acetone was determined by the method of Levine and

TABLE I. Effect of Growth Hormone on Nitrogen and Acetone Body Excretion of Normal and Adrenalectomized Fasted Rats.

	mg/100 g/24 hr	
	Range	Mean ± S.E.†
Nitrogen		
Normal (12)*	48.5–79.1	60.3 ± 2.4
Normal + G. H. (14)	34.0–70.0	53.7 ± 3.2
Adrenalectomized (12)	51.0–65.3	55.5 ± 2.4
Adrenalectomized + G. H. (22)	35.5–62.7	45.0 ± 4.1
Acetone		
Normal (12)	0 –2.2	.95 ± .21
Normal + G. H. (14)	.76–4.14	1.87 ± .30
Adrenalectomized (12)	.64–1.14	.87 ± .09
Adrenalectomized + G. H. (22)	.67–3.69	1.33 ± .34

* Figures in parentheses indicate No. of animals.

† S.E. = Standard error of mean = $\sqrt{\Sigma(x-\bar{x})^2/n(n-1)}$.

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* The growth hormone used was Lot No. GH-3, which has 25% activity as compared with the standard preparation $22KR_{21}$ kindly supplied by Armour and Co. through the courtesy of Mr. Irby Bunding.

Taterka(15). The animals were divided into 8 groups, which are represented in Table I. For each experiment a series of 6 animals was used and identical experiments were repeated 2 to 4 times.

Results. I. *Effect of growth hormone pretreatment in fasted normal and adrenalectomized rats.* A) *Normal*: As seen from Table I, the fasted normal rat had a mean 24 hours urinary nitrogen excretion of 60.3 ± 2.4 mg/100 g and 0.95 ± 0.21 mg/100 g of acetone. Pretreatment with growth hormone caused a significant increase in acetone body excretion ($P < .05$) to 1.87 ± 0.30 mg/100 g and a moderate decline in nitrogen excretion to 53.7 ± 3.2 mg/100 g, which is not statistically significant. This failure of growth hormone to reduce materially the nitrogen excretion of the fasted normal rat is in accord with a statement of Bennett and coworkers(4) that "the effect of these hormones upon the nitrogen excretion of the fasted rat is not as striking as their effect upon the nitrogen excretion in the fed rat." Similarly, Harrison and Long(3) state "that when saline A.P.E., rich in growth factor was administered on the first and second day of fasting, the nitrogen excretion for the second day is not significantly less than that observed in saline controls." The increase in acetone body excretion elicited by this growth hormone preparation is not of the same magnitude as that previously reported by others(3,4). This difference is probably due to differences in the extract, dosage levels and experimental design.

B) *Adrenalectomized.* The saline treated fasted adrenalectomized rat excreted 55.5 ± 2.4 mg/100 g of nitrogen. This is not significantly less than the normal which is in accord with the findings of Ingle(16). Also, the acetone body excretion 0.87 ± 0.09 mg/100 g was almost identical with that of the normal controls. A similar type of result has been reported from *in vitro* work(17) in which it was shown that ketone body production by liver slices from normal and adrenalectomized rats does not differ. After growth hormone administration nitrogen excretion was 45.0 ± 4.1 mg/100 g and acetone body excretion was 1.33 ± 0.34 mg/100 g, neither of which are significantly different from the untreated adrenalectomized rat. The failure of growth hormone to increase acetone excretion in the adrenalectomized rat is in accord with the findings of some authors(8-10) but not with that of others(4-7). As stated above this may be due to differences in the type of extract or the experimental design.

TABLE II. Effect of Growth Hormone on Glucose, Nitrogen, and Acetone Body Excretion of Normal and Adrenalectomized Fasted Phlorhizinized Rats.

	mg/100 g/24 hr	
	Range	Mean ± S.E.†
	Glucose	
Normal (24)*	178–616	381 ± 21.9
Normal + G. H. (12)	98–391	228 ± 30.1
Adrenalectomized (22)	102–232	144.5 ± 12.1
Adrenalectomized + G. H. (21)	43–284	137 ± 12.0
	Nitrogen	
Normal (24)	75–143	115.7 ± 4.3
Normal + G. H. (12)	42–104	69.8 ± 5.0
Adrenalectomized (22)	13– 84	50.1 ± 4.1
Adrenalectomized + G. H. (21)	34– 80	52.0 ± 2.4
	Acetone	
Normal (24)	1 –32	14.9 ± 6.3
Normal + G. H. (12)	.2–31	8.7 ± 8.9
Adrenalectomized (22)	0 –11.7	2.7 ± .76
Adrenalectomized + G. H. (21)	.5– 9.0	2.5 ± .55

* Figures in parentheses indicate No. of animals.

† S.E. = Standard error of mean =

$$\sqrt{\Sigma(x-\bar{x})^2/n(n-1)}.$$

II. *Effect of growth hormone pretreatment in fasted phlorhizinized normal and adrenalectomized rats.* A) *Phlorhizinized normal.* The normal phlorhizinized animal excreted 381 ± 21.9 mg/100 g of glucose, 115.7 ± 4.3 mg/100 g of nitrogen, and 14.9 ± 6.3 mg/100 g of acetone per 24 hours (Table II). The values for glucose and nitrogen excretion are not significantly different than those reported by Winters *et al.*(12) using a similar technic. There are, however, quantitative differences between these results and those reported by Wells and Kendall(18) and Segaloff and Many(19). These differences are probably the result of differences in procedure, batch of phlorhizin and strain of rat. Pretreatment with growth hormone depressed markedly the excretion of glucose to 228 ± 30.1 mg/100 g and nitrogen to 69.8 ± 5.0 mg/100 g. There was, however, no significant alteration of the rate of acetone body excretion (8.7 ± 8.9 mg/100 g) (Table II). This marked reduction in nitrogen excretion of the phlorhizinized animal is in accord with recent thought that the greatest apparent effectiveness of growth hormone is in situations where large amounts of amino acids are available for protein synthesis(20).

B) *Phlorhizinized adrenalectomized.* The phlorhizinized adrenalectomized rat excreted 50.1 ± 4.1 mg/100 g of nitrogen, 144.5 ± 12.1 mg/100 g of glucose and 2.7 ± 0.76 mg/100 g of acetone in 24 hours. The values for glucose and nitrogen excretion are almost identical with those reported by Wells and Kendall(18) for the phlorhizinized saline treated adrenalectomized rat. The marked lowering of the glucose, nitrogen, and acetone body excretion in this preparation as compared to the normal is also in accord with results of other investigators(18,19,21). After administration of growth hormone, glucose excretion was 137 ± 12 mg/100 g, nitrogen excretion was 52.0 ± 2.4 mg/100 g, and acetone excretion was 2.5 ± 0.55 mg/100 g. These values are identical with those obtained in adrenalectomized animals not receiving growth hormone (Table II).

Discussion. The basal rate of protein catabolism as reflected in the urinary nitrogen excretion is almost identical in the fasted normal or adrenalectomized and the phlorhizinized adrenalectomized animal and is not influenced by growth hormone (Tables I and II). On the other hand in the normal rat where the rate of protein breakdown was augmented by phlorhizination, growth hormone was markedly effective in causing nitrogen retention and a proportional decrease in glucose output. These results are in complete agreement with those previously reported for the fed phlorhizinized dog(11).

The results of this study suggest further that the effect of growth hormone on ketogenesis is independent of its nitrogen retaining effect. On the one hand, in normal rats a nonsignificant change in nitrogen excretion was accompanied by a significantly increased acetone body excretion. On the other hand, in phlorhizinized fasted rats there was a marked reduction in urinary nitrogen with no change in acetone body excretion. This concept is supported by reports that pituitary preparations of equivalent growth hormone potency have different effects on ketogenesis by liver slices and on fat mobilization(22,23).

In addition, the ketogenic activity of growth hormone apparently requires the presence of the adrenal cortex so that even under the powerful stimulus of phlorhization growth hormone pretreatment did not increase the acetone body excretion of fasted adrenalectomized rats. This is supported by studies on hypophysectomized rats in which it was shown that the injection of growth hormone within 15 days after hypophysectomy repairs the ketogenic defect of liver slices, whereas growth hormone is without apparent effect after 50 days unless cortisone is administered simultaneously with it(22). These interpretations are also in accord with the findings that the liver fat is not increased by growth hormone preparations in either normal or adrenalectomized mice unless the animals are pretreated with ACTH or cortisone(23).

The relationship of these actions of growth hormone to its diabetogenic action is not well understood. Other workers have shown that in fed alloxanized(24) or ACTH treated(25) rats, glycosuria is augmented by growth hormone. Conversely, the present study shows that in the phlorhizinized fasted rat, glycosuria is diminished by pretreatment with growth hormone, due apparently to decreased gluconeogenesis from protein. The explanation for this difference may be that the nitrogen retaining effect of growth hormone with its associated diminution in gluconeogenesis requires a high level of insulin production.

Another explanation has been suggested, namely, that a relative preponderance of catabolic activity presumably by increased adrenal steroid levels is necessary for the diabetogenic action of growth hormone(25). However, recently we showed that the blood sugar of the adrenalectomized dog is raised by growth hormone(26), presumably due to inhibition of peripheral glucose uptake. It seems that all experimental conditions under which a diabetogenic action of growth hormone has been demonstrated in the rat, have in common an increase in available glucose either by gluconeogenesis in the liver or from exogenous sources. In the phlorhizinized fasted rat, the action of growth hormone in inhibiting peripheral glucose uptake cannot be demonstrated since peripheral glucose utilization is already at a minimum. Therefore, under these conditions its effect in reducing hepatic gluconeogenesis from protein predominates. On the other hand in the partially pancreatectomized or ACTH treated rat the growth hormone induced inhibition of gluconeogenesis from protein is interfered with and the peripheral action of growth hormone predominates. This latter action then appears as an increase in urinary glucose output. It can be concluded then that since growth hormone has a dual function the apparent action will depend to a large extent on the metabolic status of the animal at the time of administration.

Conclusions. 1. Growth hormone causes a marked reduction in urinary nitrogen output in the fasted phlorhizinized normal rat but does not have this effect in either the phlorhizinized adrenalectomized or the unphlorhizinized normal or adrenalectomized animal. 2. In view of the ketogenic effect of growth hormone in the normal fasted rat and the absence of this effect in either the adrenalectomized, the phlorhizinized normal or phlorhizinized adrenalectomized rat the hypothesis is advanced that the ketogenic action of growth hormone is independent of its nitrogen retaining action and apparently requires the presence of the adrenal cortex for its appearance. 3. Growth hormone caused a marked reduction of glycosuria in the phlorhizinized fasted rat. This together with the reported increased glycosuria of alloxanized or ACTH treated rats after growth hormone lead to the conclusion that the diabetogenic action of growth hormone is indicative of a relative preponderance of the inhibitory action of growth hormone on peripheral glucose uptake over its action in reducing gluconeogenesis from protein in the liver.

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A Modified Bristle Flowmeter for Measuring Phasic Blood Flow.* (20293)

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Previous studies of venous return in this laboratory(1,2) have impressed us with the difficulty of recording phasic changes of venous flow with accuracy by the usage of flowmeters currently available. Since a number of problems requiring the study of phasic flow still remain, we have for some time attempted to adapt the Holzlöhner "stream-bristle" cannula for such studies(3-5). This instrument, based upon the blood velocity recorders of Chauveau-Lortet and de Burgh Daly(6), is particularly useful in that it measures both velocity and volume flow by recording electrically the deviation of a fine glass bristle suitably inserted into the blood stream by means of a specially constructed cannula. Further advantages are: 1) high fidelity response (500 cycles/sec.). 2) High and variable sensitivity. 3) Linear calibration. 4) Negligible resistance to blood flow. 5) Compactness. 6) Nonturbulence over the physiological flow range. 7) Registration of backflow.

One of the authors, (G.A.B.) who had the privilege of working with Holzlöhner in developing and constructing the stream-bristle cannulae, was aware of the difficulties in making the fine platinum-glass bristles. The recent availability of the RCA 5734 transducer vacuum tube, however, permitted the authors to construct a suitable modification of a bristle flowmeter of the Holzlöhner type.

Fig. 1 shows a schematic diagram of the modified stream-bristle cannula. The T-shaped cannula consists of 3 brass parts, (X,Y,Z) which can be assembled by threaded joints. The transducer tube (5734) is mounted in section X. Provision for pressure recording and for removing air bubbles is made by a side tube attached to section Y. Section Z is the cannula of fixed diameter introduced into the blood vessel. It can be turned to assure maximal bristle sensitivity. A very thin stainless steel filament is soldered to the plate-pin (P) and serves as a bristle (S) protruding into the blood stream. Deviation of the plate-pin causes the resistance of the RCA 5734 tube to vary. With the circuit connections shown in Fig. 2, this resistance variation in the transducer (T-1) gives rise to an electrical signal linearly proportional to flow. The signal is amplified by tubes T-2 and T-3. The output of the latter actuates a D-C power amplifier driving a mirror oscillograph. Relative freedom from drift is accomplished by battery plate and filament supplies, and the differential nature of the amplifier stage (T-2 and T-3).

Numerous flowmeter designs have been described in the literature, which have not satisfied the demands under actual animal experimental conditions. Holzlöhner's flowmeter

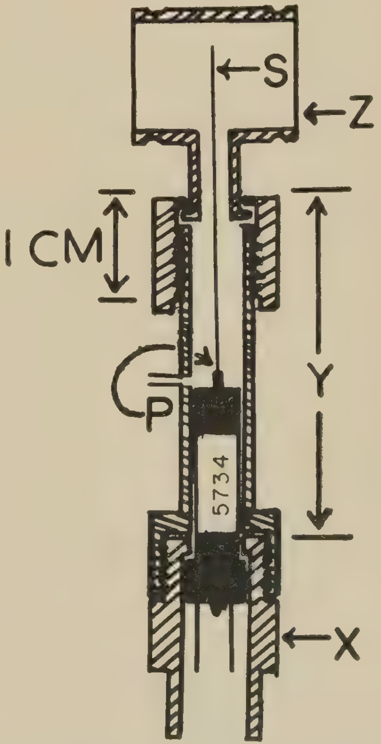

FIG. 1. Diagram of stream-bristle cannula. Description in text.

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* Project supported by Life Insurance Medical Research Fund.

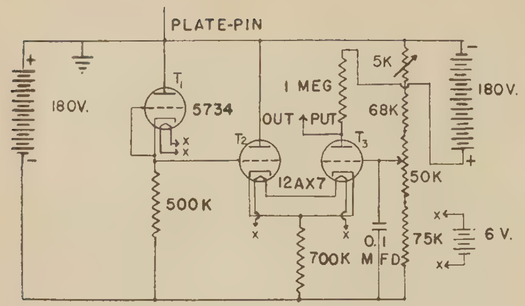

FIG. 2. Electronic circuit of RCA 5734 stream-bristle flowmeter. Description in text.

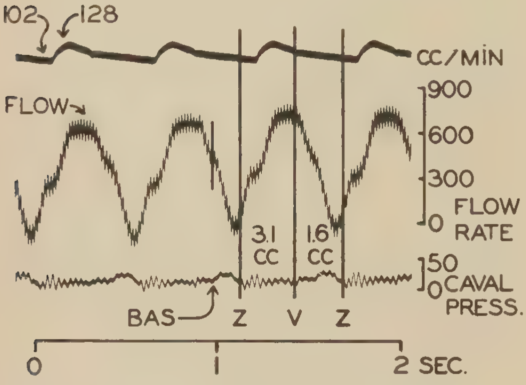

FIG. 3. Effect of cardiac movement on venous return. Tracings from top to bottom: Pressure in abdominal aorta in mm Hg. Superior vena cava flow. Pressure in superior cava in mm H_2O. Time 1/60 sec. in flow tracing.

has met these critical requirements, and the present modification, which has the same advantages, has been employed with equal success in this laboratory for blood flow determinations.

Low resistance to blood flow makes the stream-bristle cannula particularly suitable for an accurate analysis of the dynamics of venous return. Fig. 3 shows a segment of an original record taken with this modified bristle flowmeter. It depicts the changes of superior vena cava flow during a normal cardiac cycle in an open chest dog, when artificial respiration is stopped a few seconds for recording purposes. Simultaneous recording of the pressure pulses in the superior cava and abdominal aorta permit precise timing of the acceleration and deceleration of venous return. With the beginning of atrial systole (BAS), inflow is markedly slowed down from a rate of 500 cc/min to very low rates or even zero flow. At the end of atrial systole, flow begins to accelerate rapidly (Z—point in atrial pressure curve, occurring prior to closure of tricuspid valves). The inflow rate reaches its maximum (800 cc/min) prior to the opening of the tricuspid valves (V—point in the atrial pressure curve). Thereafter, flow is slowed down to varying degrees, and the cycle is repeated when flow again reaches its minimum during atrial systole. By measuring the area under the flow curve, it is possible to determine the amount of volume flow during different parts of the cardiac cycle. It is noted that inflow into the right atrium is almost twice as much during ventricular systole, when the tricuspid valves are closed and no blood can leave the atrium (3.1 cc from Z to V), than during ventricular diastole, when the tricuspid valves are open and atrium and ventricle form a common cavity (1.6 cc from V to Z). This demonstrates the active influence of ventricular contraction on the forward movement of blood in the central veins. The marked increase of venous return during the period when the tricuspid valves are closed, and blood cannot enter the ventricle, may be explained by the descent of the atrio-ventricular plane during ventricular systole, passively distending the atria for greater accommodation of blood. Changes of inflow from the inferior cava are expected to be similar, as one may conclude from simultaneous flow measurements with two Pitôtmeters in both superior and inferior cava(7).

Summary. A new type of electrically recording bristle flowmeter is described, which permits measurement of volume flow of blood and its phasic changes. By recording superior vena cava flow, direct evidence is presented that the greatest acceleration of blood and a marked volume inflow into the right atrium occurs during ventricular systole, when the tricuspid valves are closed. This demonstrates the active influence of ventricular contractions on venous return.

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Mechanism of Ovarian Control of Hypophyseal Gonadotrophic Function in Guinea Pig. (Experiments with Intrasplenic Grafts). (20294)

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Removal of one ovary does not affect the size of subsequent litters, *i.e.* the number of maturing follicles and of ova shed. Thus the conclusion was reached that follicular development is regulated by the production in the body of a fixed quantity of extra-ovarian substances ("law of follicular constancy") (1).† These extra-ovarian substances can now be identified with the pituitary gonadotrophins. On the other hand, it has been suggested that not only is follicular development quantitatively regulated by the production of these extra-ovarian substances but also that their use, or consumption, in the ripening follicles partakes in the maintenance of the normal ovarian and hypophyseal relationship; ovarian function would depend upon a balance between production and consumption of gonadotrophins(2). This concept though purely hypothetical was based on the experimental statement that an abnormal endocrine function of the ovary is observed after the removal of one entire ovary and the greater part of this second ovary. In similar animals with an ovarian fragment, or remnant, cystic hyperplasia of the endometrium, penetration of glands into the myometrium, uterine and extra-uterine tumors can be observed when the experiments are sufficiently prolonged(3). In the ovarian fragment signs of an abnormal follicular development may be present, as lutein cysts, occasionally also an hemorrhagic one. It was then assumed, though rather vaguely, that the overthrow of the normal ovarian hypophyseal relationship is due, under the described experimental conditions, to an imbalance between production and consumption of gonadotrophins in the ovarian remnant.

The concept of a regulatory influence exercised by the ovary on the hypophysis by the consumption of gonadotrophins was discarded when the control of the hypophysis by ovarian estrogens became a definitely established fact (4-6). The quantitative and timing conditions of this control are still under discussion (7,8). However, more recently the problem has been discussed on new experimental lines such as work with intrasplenic ovarian grafts in the rat; the important statement was made that the hypophysis of the female rat with both ovaries grafted into the spleen is not identical to that of a castrate in so far as it contains less gonadotrophins than the hypophysis of the castrated rat(9). This non-identity is shown also in the absence of castration cells in the hypophysis of castrated animals with intrasplenic ovarian grafts(10). These results were interpreted favoring the concept that consumption of gonadotrophins was a regulatory factor of the functional condition of the hypophysis(9). On the other hand, experiments made in this department have shown that in the guinea pig a small ovarian fragment *in situ* is capable of counteracting production of hemorrhagic follicles in the intrasplenic graft(11-13) even when follicular development in the fragment is very small, *i.e.*, when the assumed consumption of gonadotrophins apparently did not count(14).

In view of these discrepancies the following 2 comparative sets of experiments were made.

A. *Intrasplenic transplantation of variable*

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† Our concept was but a modernized and more "experimentalized" version of Hunter's, Heapes's, Sand's and Hammond's.

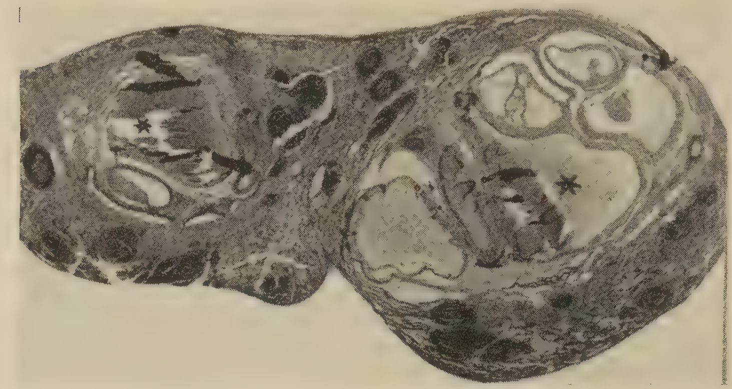

FIG. 1. Both entire ovaries grafted into the spleen; 2 months after grafting; autograft. *Hemorrhagic follicles in both ovaries*‡. Various *cystic* Graafian follicles; no corpora lutea (though they may be present). × 10 (CLVI. 8).

quantities of ovary. Whereas in our former work only one ovary was grafted into the spleen, the second one being excised, in new work *both* whole ovaries were grafted into the spleen. Animals were necropsied 2 months after transplantation. Only those experiments have been included in the results in which both ovaries survived in the spleen. There were 36 animals with 2 ovaries in the spleen (5 series operated on different occasions); in 24 out of these 36 animals (67%), hemorrhagic follicles demonstrating uncontrolled hypophyseal activity were found. In many cases both the ovaries grafted had hemorrhagic follicles (Fig. 1). The frequency of hemorrhagic follicles in experiments with one ovary in the spleen was higher; with one ovary in the spleen, 90-100% of grafts with hemorrhagic follicles were found. This was also the case in 3 series of 22 animals (out of the total of 5 series) with 2 ovaries in the spleen; 19 out of these 22 animals, or 86% had hemorrhagic follicles. The smaller average in the whole group of 36 animals with 67% was due to a failure in 2 series of 14 animals with 2 ovaries in the spleen; only 5 of these had hemorrhagic follicles. The most probable is that a tiny fragment of ovary was involuntarily left in the body of some of these animals. There was among the group of 36 animals a series of 9 animals in which besides the 2 autografts, 2 homoiografts were also made simultaneously but did not take. Cystic condition of Graafian follicles, hemorrhagic or not, was frequent. Corpora lutea were probably less frequent than when only one ovary was grafted. The above results give evidence that both ovaries present simultaneously in the spleen are as incapable as one ovary of controlling the gonadotrophic function of the hypophysis. Certainly, the results do not disprove the concept of a regulatory influence of the ovary on the hypophysis by the means of consumption of gonadotrophins; but they show clearly that such an influence is not sufficient to maintain the gonadotrophic hypophyseal function on a normal level.

B. *Simultaneous transplantation of one ovary into the spleen and of the second one into the kidney* (Fig. 2 and 3). Under these experimental conditions it is to be supposed that consumption of gonadotrophins will be the same as with two ovaries in the spleen, whereas estrogens from the intrarenal graft will reach the general circulation. Animals were again necropsied 2 months after transplantation and only those animals were considered in which grafts survived both in the spleen and in the kidney. There were 10 such animals and there was none with hemorrhagic follicles in the intrasplenic graft.‡ Cystic condition of Graafian follicles was less frequent.

Summary. Hemorrhagic follicles in intrasplenic ovarian grafts in castrated guinea pigs

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‡ We have found hemorrhagic follicles, exceptionally, even in intrarenal ovarian grafts (see 16, p. 178). The intrarenal graft, like the intrasplenic one, is incapable of affording a complete control of the hypophysis. This is likewise true for subcutaneous ovarian grafts(3,17).

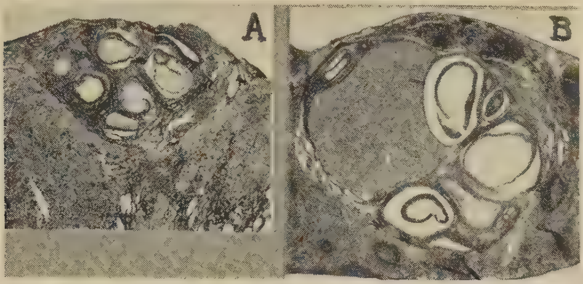

FIG. 2. One ovary grafted into the kidney (A); the whole ovary slipt out to the surface of the kidney. The second ovary grafted into the spleen (B). 2 months; autograft. *No hemorrhagic follicle in B.* No cystic Graafian follicles; very large corpus luteum in B. × 10. (CXXXVIII. 121).

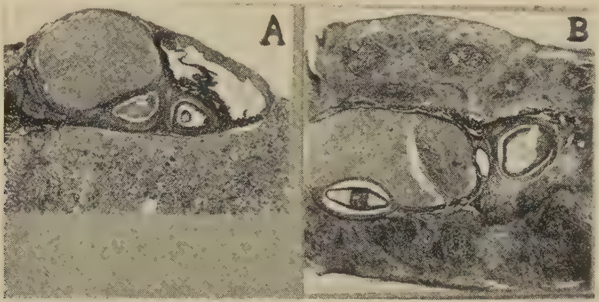

FIG. 3. One ovary grafted into the kidney (A); the greater part of the ovary is on the surface of the kidney. The second ovary grafted into the spleen (B). 2 months; autograft. *No hemorrhagic follicle in B.* No cystic Graafian follicles. [The cystic formation in A is Wolffian; this we consider as indicative of an ovarian-hypophyseal imbalance(15), though of small degree.] Corpora lutea both in A and B. × 10. (CXXXVIII. 133).

appear irrespectively whether one or 2 ovaries are grafted into the spleen. On the contrary, hemorrhagic follicles fail to appear in an intrasplenic graft of the castrated guinea pig when the second ovary is grafted into the kidney. These experiments give definite evidence that the ovarian control of the hypophyseal gonadotrophic function depends not only, if at all, on consumption of gonadotrophins but certainly also on ovarian hormones which reach the general circulation.

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Prevention of Blood Coagulation by Antibiotics *in vitro.* (20295)

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(Introduced by Reuben Kahn.)

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Antibiotics probably do not affect blood coagulation *in vivo*(1-4). The concentrations of antibiotics which can be attained in the blood of animals or man are limited. It is possible to demonstrate *in vitro,* however, that in suitable concentrations several antibiotics are anticoagulants.

Experimental. Streptomycin sulfate (Parke Davis), neomycin sulfate (Upjohn), oxytetracyclene glycinate ("terramycin" Pfizer), Q-19 (Upjohn), potassium penicillin G (Pfizer), and sodium citrate were each dissolved in saline. Then 0.1 ml of each solution containing one of these agents was mixed with 1.0 ml fresh human blood to give final concentrations ranging from 0.5 to 50 mg per ml. Bloods containing antibiotics were tested for clotting time by the Lee-White method for one hour, refrigerated (4°C) for 23 hours, and examined again for clotting. The bloods containing antibiotics were examined in a Beckman pH meter. The pH of control bloods was adjusted to that of samples containing antibiotics. At the end of 24 hours, $CaCl_2$ was added to unclotted samples to give concentrations exceeding that of the antibiotic.

The results are summarized in Table I. They were reproduced with each antibiotic in many different samples of blood. As an anticoagulant, oxytetracyclene was at least 8 times more effective than any of the other antibiotics. Glycinate was no anticoagulant and did not influence clotting when added to the other antibiotics. Slight hemolysis was sometimes observed in unclotted blood 24 hours after adding oxytetracyclene but not other antibiotics. Chloramphenicol did not dissolve in the desired concentrations in water. The anticoagulant action of streptomycin and penicillin was reversed by excess amounts of $CaCl_2$. It is suggested, therefore, that these agents prevented clotting by binding calcium. Calcium did not reverse the anticoagulant activity of neomycin or oxytetracyclene. The prothrombin time (Quick's method) of blood containing 50 mg per ml of streptomycin was 56 seconds and of the same blood without streptomycin was 12.7 seconds; it was prolonged indefinitely by 100 mg per ml of neomycin. Overman and Wright(5) have also observed that streptomycin increases the prothrombin time and clotting time and suggested that streptomycin inhibited thromboplastin. The pH of bloods containing anticoagulant concentrations of antibiotics ranged from 6.9 (neomycin) to 8.8 (oxytetracyclene). There was no delay in clotting of bloods with pH ranging from 6.9 to 8.8 in the absence of antibiotics.

TABLE I. Minimum Concentration of Antibiotics and Sodium Citrate (mg/ml of Blood) Which Prevented Clotting.

Sodium citrate	1
Oxytetracyclene ("terramycin") glycinate	2.5
Neomycin sulfate	20
Streptomycin sulfate	25
Potassium penicillin G	30
Q-19*	Clotted with 50

* An antibiotic of the polymyxin type(6). Concentrations of 40 mg/ml delayed clotting for 1 hr.

Summary. When examined *in vitro,* streptomycin, penicillin, oxytetracyclene ("terramycin") and neomycin were anticoagulants only when used in greater concentrations than can be attained *in vivo.*

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Action of an Antiviral Mold Filtrate against MEF1 Poliomyelitis Virus in Mice. (20296)

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We have reported on a penicillium mold filtrate designated serially as M5-8450 (hereinafter called 8450) which has considerable chemoprophylactic action coupled with very slight chemotherapeutic action against MM and Semliki Forest viruses in white mice(1). Protective action against 100 LD_{50} or more of either virus as injected subcutaneously may be obtained by injecting mice intraperitoneally with a single dose of 0.05 ml up to 0.25 ml of 8450 provided filtrate antedates virus by about 24 hours. Other combinations of routes and time of injection of virus and filtrate may be used with varying degrees of effectiveness with the exception that we have obtained negative results whenever (A) 8450 is given orally, or (B) virus is given intracerebrally. We have not observed any refractoriness in mice injected with a series of doses of 8450, also have observed no substantial antiviral immunity in mice once protected from virus by suitable treatment with 8450, nor have we found any indication that this agent neutralizes virus *in vitro*.

We wish to present here some further results of the activity of mold filtrate 8450 in mice infected peripherally with type 2 poliomyelitis virus MEF1.

Materials and methods. The MEF1 strain of poliomyelitis virus was obtained as mouse brain and cord from Dr. William McD. Hammon of the University of Pittsburgh. Typing serum comprising hyperimmune monkey antiserum in the frozen state was obtained through the kindness of Dr. Howard A. Howe of the Johns Hopkins University. Young cotton rats of 40 to 50 g weight and subsequently 7-day-old hamsters were used to passage serially a special line of the MEF1 virus preliminary to its use in chemotherapy tests in mice. The mice utilized were albino Swiss of about 12 g in weight each. 8450 mold filtrate has been referred to(1).

Procedure and results. The MEF1 virus was kept in stock as Swiss mouse brain material removed at autopsy from mice showing various degrees of paralysis following intracerebral injection of seed virus. This stock mouse brain virus was passaged 6 times intracerebrally through 40 to 50 g cotton rats. Sixth cotton rat passage virus in addition to producing paralysis and CNS lesions was neutralized by type 2 antiserum (lot E-397) referred to above as follows. Three decimal dilutions of virus, as judged from previous virulence tests in mice to contain 1, 10, and 100 LD_{50} of virus in ½ the usual intracerebral dose, were mixed with antiserum diluted 1:5 and along with controls let stand 2 hours at 4°C and 2 hours at room temperature, then injected intracerebrally in doses of 0.03 cc into groups of 8 Swiss mice of 15-17 g weight. The results as shown in Table I indicate satisfactory neutralization of MEF1 stock virus. Sixth cotton rat passage of MEF1 virus was passaged intracerebrally in series through 7-day-old hamsters. Hamster passage 21 was neutralized to approximately the same extent by a portion of the same antiserum lot referred to above and the details similar to those just indicated need not be included here. Intermediate hamster passages 12, 15, 17, and 20 in addition to 21 will be utilized as indicated below.

TABLE I. Neutralization Test of MEF1 Virus in Mice with Type 2 Hyperimmune Monkey Serum.

No. LD_{50} inoculated	With antiserum	Controls
100	1/8	8/8
10	0/7*	7/7*
1	0/7†	4/8
.1	—	0/8

* 1 death due to intercurrent disease.
† 1 " " " trauma.
Numerator of fraction = No. dead; denominator = No. used.

Before hamster passage, MEF1 virus either as mouse brain or cotton rat brain material, although virulent peripherally for suckling mice, was practically innocuous peripherally for 12 g Swiss mice. During hamster passage,

TABLE II. Effect of Mold Filtrate 8450 in Mice Infected with Hamster Passaged Poliomyelitis Virus MEF1.

.2 ml MEF1 virus	.25 ml × 4 of 8450	Virus dilution	Day of death or survival (S) for 30 days	
			Treated mice	Control mice
Hamster passage 12 i.p.	i.p.*	10^{-1}	5, (7), 11, S, S, S, S, S, S, S	3, 3, 5, 6, 6, 8, 14, S, S, S
Hamster passage 15 i.p.	”	”	3, 20, S, S, S, S, S, S, S, S	3, 3, 4, 4, 5, 5, 5, 6, 6, 9
Hamster passage 17 i.p.	oral	”	4, 4, 4, 4, 4, 6, 7, 7, S, S	4, 4, 4, 4, 4, 5, 6, 6, 6, 6
		10^{-2}	8, 15, S, S, S, S, S, S, S, S	6, 7, 7, 8, 13, S, S, S, S, S
	subq.	10^{-1}	3, 3, 4, 6, 6, 6, 6, 6, 14, S	
		10^{-2}	4, 6, 7, 9, 13, S, S, S, S, S	
Pool of hamster passages 17, 20, & 21 i.p.	i.v.	10^{-1}	6, 6, 9, 15, 20, S, S, S, S, S	3, 3, 3, 3, 3, 6, 6, 6, 8, S
		10^{-2}	S, S, S, S, S, S, S, S, S, S	8, 9, 13, S, S, S, S, S, S, S
	i.m.	10^{-1}	3, 3, 6, 6, 6, 6, 6, 6, 8, 17	
		10^{-2}	6, S, S, S, S, S, S, S, S, S	
	i.p.*	10^{-1}	6, 10, 17, S, S, S, S, S, S, S	
		10^{-2}	S, S, S, S, S, S, S, S, S, S	
As above i.m.	”	10^{-1}	6, S, S, S, S, S, S, S	6, 6, 6, 10, 10, S, S, S
		10^{-2}	S, S, S, S, S, S, S, S	S, S, S, S, S, S, S, S
As above i.v.	”	10^{-1}	3, 3, 3, 3, 3, 6, 6, 7, S	3, 3, 3, 6, 6, 6, 6, 6, 7, 9
		10^{-2}	3, 6, 22, S, S, S, S, S, S, S	3, 3, 3, 6, 6, 7, 11, 16, S, S

Treatment with 8450 was 1 dose/day for 4 days beginning 1 day before virus inj. In experiments in which both 8450 and virus were given intrap. and marked *, the dose of 8450 was omitted on the day virus was inj. (7) indicates mouse dead of intercurrent infection in 7 days. In 1 exp. we used 8 mice/group and in others 10. Nearly all mice that died were observed to have paralysis in one or more extremities for some time before death.

a considerable degree of peripheral virulence developed for 12 g mice, a fact noted heretofore by others. This appeared to offer a test method superior to tests in suckling mice whereby filtrate 8450 could be tested against peripherally injected poliomyelitis virus before resorting to tests in monkeys.

In Table II are shown the results of several chemoprophylactic tests in 12 g mice of 8450 given in a series of 4 doses, the first dose of which was given the day before MEF1 poliomyelitis virus was injected. (This particular lot of 8450 when injected as a single intraperitoneal dose of 0.25 ml the day before infection gave full chemoprophylaxis against 100 LD_{50} of MM virus injected subcutaneously in tests in mice not shown here.) The virus used in experiments in Table II comprised passage 12, 15, 17, and a pool of 17, 20, and 21, from a series of passages conducted in hamsters of 7 days age.

It may be observed that 8450 given to mice intraperitoneally accomplished demonstrable chemoprophylaxis against MEF1 virus also given intraperitoneally. In tests of this sort in which both filtrate and virus were given intraperitoneally, filtrate dosage to the mice was omitted on the day of virus injection. Administration of filtrate 8450 orally or subcutaneously had practically no effect on mice injected intraperitoneally with MEF1 virus, however, when the filtrate was injected intravenously into mice there was apparent chemoprophylaxis against MEF1 virus injected intraperitoneally. 8450 did not appear to be effective when injected intramuscularly. A trace of effectiveness was observed when virus was given intramuscularly (virulence not high enough) and filtrate was given intraperitoneally, and also when virus was given intravenously and filtrate was given intraperitoneally. It appears that some further tests of this last type should be done since the virus appeared a little more virulent intravenously than anticipated. Also, it remains to be determined whether filtrate lots which are active against MM and Semliki Forest viruses in mice (both activities apparently due to the same principle) are necessarily active to the extent described against MEF1 virus.

Summary. 1. A penicillium mold filtrate designated as 8450, and reported previously to have chemoprophylactic and chemotherapeutic activity against MM and Semliki

Forest virus in mice, has been found to have demonstrable chemoprophylactic effects in 12 g mice infected peripherally with a special hamster passaged line of MEF1 poliomyelitis virus. 2. Experiments are under way to find out (A) if stronger chemoprophylaxis can be obtained, (B) if more prolonged action can be obtained, and (C) if oral activity can be attained by 8450 against poliomyelitis virus.

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A Quantitative Comparison of Antibody Produced by X-Radiated and Normal Rabbits.* (20297)

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The antibody response can be divided into a brief initial radiosensitive phase and a second radioresistant phase which occupies the remainder of the period of response(1a,b,c,d). Once initiated, antibody production is little affected by large doses of whole body radiation in spite of the fact that lymphatic tissues, bone marrow, and spleen—all thought to be important in the production of antibody—are extremely radiosensitive. Several explanations of this observation have been given: first, the radiosensitive tissues might function only in the brief, initial phase of the antibody response and that the actual synthesis of antibody might take place in radioresistant tissues(1c); second, since even large doses of radiation do not destroy all of the radiosensitive cells in the body it is possible that the small number of these cells remaining might carry on the full antibody response; and third, that there are radioresistant reserve tissues capable of carrying out antibody formation once it has begun in spite of damage to radiosensitive tissues(2).

In view of the above, it seemed worthy to investigate whether the character of antibody molecules might be altered in radiated rabbits. The only readily demonstrable differences between antibodies are immunochemical in nature (3a,b,c,d) and these differences are based on the average behavior and reactivity of all the different kinds of antibody to the same antigen. The present report deals with a comparison of the immunochemical characteristics, as evaluated by the quantitative precipitin reaction and the initial combining ratio of antibody to antigen, of antibodies to bovine gamma globulin[§] (BGG) and bovine serum albumin[||] (BSA) formed in X-radiated and normal rabbits.

Materials and methods. Male albino rabbits weighing from 2 to 2.3 kg were immunized and radiated as indicated in Table I. A single intravenous injection of either I^{131} labelled BGG (I*BGG), 30 mg/kg, or I^{131} labelled BSA (I*BSA), 15 mg/kg, was given to each animal. Preparation of the iodinated antigens has been previously described(4a,b). Two hundred and twenty KV whole body X-radiation was given as previously described(1c) in either 400 r ($LD_{5\text{-}30}$) or 800 r ($LD_{75\text{-}30}$) doses. Antigen concentrations in the blood were determined by protein bound radioactivity(4b) and bleedings for antibody analyses were made 3 days after elimination of all antigen at which time serum antibody concentrations were usually at a maximum. In order to have sufficient serum for quantita-

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[§] Armour and Co. Fraction II bovine serum.

[||] Armour and Co. crystalline bovine serum albumin.

TABLE I. Relation of Immunization, Radiation, and Antibody Response.

Animal No.	Immunization and radiation	Antibody bleeding days after inj.	Serum antibody conc., μg antibody N/ml
381, 382	I*BGG 5 hr after 400 r	13	7.5
266–269	” 48 ” ” ” *	14	25.1
295	” 3 days before 800 r	13	72.1
423, 301	” ” ” ” ”	13	34.2
408	” 1 hr ” ”	13	54.0
725, 726, 728, 734	I*BSA 48 hr ” 400 r	12–16	25.8
707, 709, 711, 712, 713	” simultaneous with 400 r	17–19	6.1
717, 720, 721, 722, 724	” 6 hr after 400 r	17–19	5.0
472, 481, 484	I*BGG—no X-ray	10	81.0
478, 490	” ” ”	10	48.0
698, 704	I*BSA— ” ”	14	38.0

* Seven injections egg albumin and edestin prior to X-ray to "protect" immune response.

tive analysis, sera from similarly treated animals with antibody concentrations at about the same level were pooled as shown in Table I. The quantitative immunochemical procedures used were those described by Heidelberger and coworkers(5). In some experiments I^{131} labelled antigen was used and its concentration was measured as previously described (6). Nitrogen was measured by the Markham modification of the micro-Kjeldahl procedure (7). The methods used for decomplementing low titer rabbit antisera with an egg albumin-anti-egg albumin (rabbit) specific precipitate has been presented(6). Gross and microscopic study of the tissues of some of the animals listed in Table I was made following the antibody bleeding. In addition the tissues of other animals receiving 400 r and 800 r whole body radiation and sacrificed 3, 5, 8, and 11 days post radiation have been studied to afford a complete picture of the post radiation tissue changes. Since the radiation changes are more clear-cut with the 800 r dose only these histologic observations will be presented.

Results. A convenient method for comparing anti-protein antibodies is to plot the ratio of antibody N/antigen N in the precipitate against the square root of the antigen nitrogen in the precipitate. The intercept of the straight line gives the initial combining ratio (2R) of antibody N/antigen N. A complete discussion of this can be found in(8). As the value for the initial combining ratio (2R) is known to change upon repeated immunization of the same animal(9), its usefulness as an absolute method in comparing antibodies produced against the same antigen is somewhat limited. Major differences in reactivity of antibodies, however, can be detected. Analysis of the decomplemented antisera from rabbits immunized with I*BGG, 5 and 48 hours re-

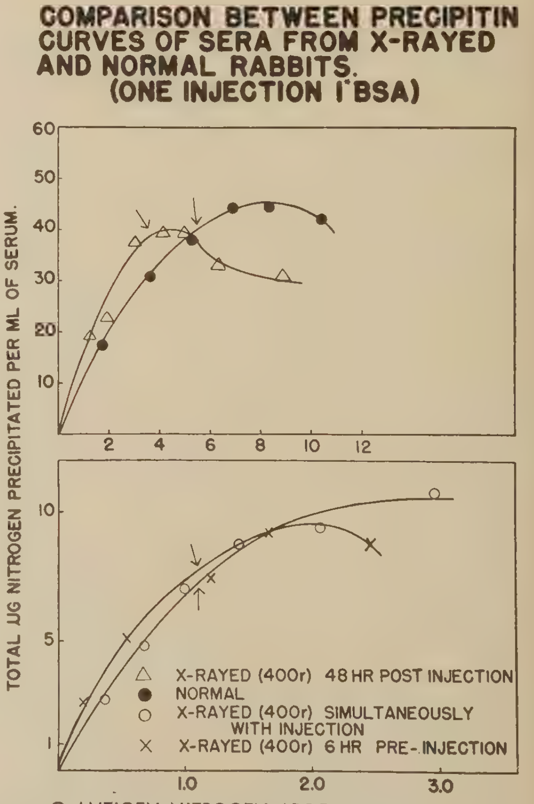

FIG. 1. The arrows indicate the equivalence point.

TABLE II. Initial Combining Ratios (2R) of Anti BSA Sera.

Exp. group	Antibody N/ antigen N = 2R
I*BSA 48 hr before 400 r	18.0
" 6 " after "	17.2
Control	16.1

spectively after 400 r whole body radiation showed precipitin curves and initial combining ratios entirely comparable with those of antisera produced by nonradiated rabbits (Reference 6, Fig. 7 and 8). As was pointed out in earlier studies(6) it is extremely important to decomplement rabbit sera before immunochemical analysis especially when the concentration of antibody is low. Failure to do this may result in erroneous values for both antibody nitrogen and initial combining ratios.

These experiments were repeated with crystalline BSA using 400 r as indicated in Table I. The results summarized in Fig. 1 indicate that the immunochemical behavior of the anti-BSA sera from radiated rabbits is essentially the same as the control. Considering that the quantitative analyses were performed on sera having a low level of antibody N the agreement is very good. Inhibition of precipitation occurs in the region of antigen excess as is to be expected. The initial combining ratios (Table II) of the above mentioned sera are the same, within the experimental errors involved.

In order to observe the effects of larger amounts of radiation 800 r was given to one group of rabbits and sera obtained during the second post radiation week when there was little morphologic evidence of recovery from the radiation injury as described below. Although these sera were analyzed without decomplementation it is obvious that their initial combining ratios would be the same as found in nonradiated controls (Fig. 2). In addition, the immunochemical behavior of the antibodies from the 800 r animals as shown by the precipitin curves closely parallels that of the controls (Fig. 2). In the animals receiving 800 r whole body radiation there was severe damage to the radiosensitive tissues. Injury to the bone marrow resulted in loss of nearly all of the hematopoietic cells leaving behind fat cells, scattered reticuloendothelial cells, and a peculiar gelatinous intercellular matrix. This destruction was well developed within 5 days and significant recovery did not begin for 2 weeks. The spleens of these animals were considerably reduced in size within a few days largely at the expense of the lymphoid tissue. Small lymphocytes were almost entirely destroyed and only small foci of large lymphoid cells and plasma cells persisted about the splenic arterioles. A moderate number of macrophages and fibroblasts appeared in and about the splenic white pulp during early post radiation period. There was a diffuse regeneration of lymphoid tissue which began during the second week post radiation but development of lymphoid follicles and appearance of small lymphocytes was not seen for several weeks. The lymph nodes at the hilus of the intestinal mesentery showed marked damage which reduced the total number of cells in the node to 10-20% of the normal. Lymphoid follicles and small lymphocytes were completely destroyed leaving a loose network of reticuloendothelial cells and fibers in which were scattered large lymphocytes, plasma cells, macrophages and fibroblasts. There was little evidence of regeneration before the middle of the second post radiation week and normal structure was not regained for a month or more. The lymphoid tissue of the appendix showed degenerative changes comparable to those seen in the nodes. The lymphoid follicles were replaced by

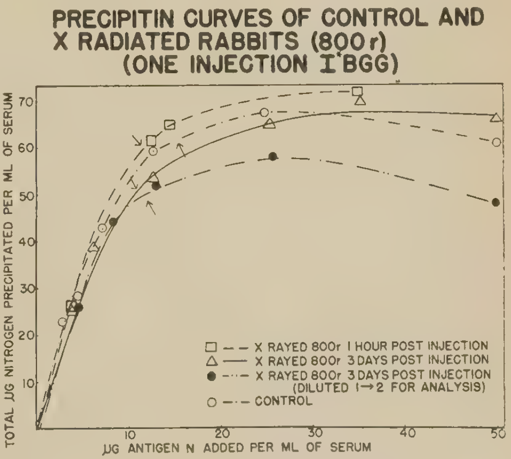

FIG. 2. The arrows indicate the equivalence point.

clumps of foamy macrophages and the amount of lymphoid tissue remaining one week after radiation could not have been more than 10% of normal. There was little evidence of recovery before the third post radiation week.

Discussion. The radiation dose of 400 r prior to or near the time of immunization was used to interfere with the early radiosensitive phase of antibody production.¶ Eight hundred r was given after the injection of antigen to allow us to study antibody formed in animals showing maximal radiation injury. With neither 400 r nor 800 r was there demonstrable immunochemical alteration in the character of the serum antibody although the concentration of the antibody was greatly reduced in those animals radiated prior to or at the time of immunization (Table I).

The observations in the 800 r group are the most dramatic. Here animals with the greatest part of their lymphoid and hematopoietic tissues destroyed, and the remainder of these tissues certainly far from normal, synthesized normal amounts of antibody which did not differ immunochemically from antibody produced by nonradiated animals. This observation is consistent with the view that the synthesis of antibody (second phase of the antibody response) may occur in radioresistant cells or tissues(1c).

Conclusions. The antibody formed by rabbits radiated with 400 r whole body radiation prior to, simultaneously with, or after immunization and by rabbits radiated with 800 r after immunization although often reduced in amount was indistinguishable from that formed by nonradiated rabbits.

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¶ The second group of animals in Table I (266-269) had several injections of egg albumin and edestin the week before radiation to increase nonspecifically the radioresistance of immune response. Antibody concentration of these sera is definitely higher than that obtained from animals not treated prior to radiation indicating some protective effect from this procedure.

On the Metabolic Alteration of Pteroylglutamic Acid.*† (20298)

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The enzymatic formation of a derivative of folic acid (FA) which promotes the rapid growth of the microorganism, *Leuconostoc citrovorum* (ATCC 8081), was demonstrated by incubating slices of rat liver with pteroylglutamic acid (PGA) in an air-equilibrated medium(2). Under similar conditions, however, homogenates of rat liver failed to form more than trace amounts of citrovorum factor (CF) from pteroylglutamic acid(3). The effectiveness of ascorbic acid in augmenting the amount of CF formed by the slices suggested that anaerobic conditions might favor the reaction. Data presented here show that satisfactory yields of CF were obtained when homogenates of liver were incubated in the presence of PGA under an atmosphere of nitrogen. Also, different tissues have been compared with respect to their capacity to form CF from PGA *in vitro* and the hepatic concentration of CF was determined with respect to time following the parenteral administration of PGA to rats previously depleted of FA.

Experimental. Weanling albino rats were depleted of FA by feeding a purified casein-cerelose diet containing succinylsulfathiazole which was described previously(2). Rhode Island Red chicks of mixed sex were fed a similar FA-deficient diet supplemented with gelatin (10%). The normal rats were maintained on a complete stock diet. The chicks and rats were used for experiments *in vitro* when signs of FA-deficiency were apparent. Like FA, *CF must be released* from the conjugated forms in which it occurs naturally before it is active for the assay organism, but liver is an excellent source of such hydrolytic enzymes(4). In the studies of the concentration of CF in the liver of rats which were injected intraperitoneally with PGA, satisfactory release of CF was obtained by autolysis of homogenates of fresh liver under toluene at 37°. The homogenates (1:4) were prepared in 0.1 M phosphate buffer of pH 6.5. Studies on the rate of release of CF showed that although autolysis for 12 hours was insufficient, maximal values were obtained after 18 hours. In this study, 10 cc aliquots of homogenates prepared from the individual livers were autolyzed for 20 hours, then steamed for 10 minutes, diluted to 25 cc and filtered. The preparation and incubation of the tissue slices was described previously(2). Homogenates were prepared in a glass homogenizing-tube with 0.1 M phosphate buffer, pH 7.0, so that 5.0 cc of homogenate contained 1 g of fresh tissue. After incubation, the tissues were steamed for 10 minutes. The filtrate of each sample was diluted appropriately for assay using *Leuconostoc citrovorum* in the medium described by Sauberlich(5). Following incubation of the 10 cc assay tubes for 18 hours at 37°, turbidity readings were made using a Klett-Summerson photoelectric colorimeter with a No. 66 filter. The barium salt of synthetic citrovorum factor was used as a reference standard; the concentration was expressed in terms of the free acid. Since synthetic CF (leucovorin or folinic acid-SF) as the free acid, was found to be 50% as active as PGA when

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† As used by Welch and Nichol(1) the term pteroylglutamic acid (PGA) has been restricted to the synthetic compound, and citrovorum factor (CF) to the substance or substances active in promoting the rapid growth of *Leuconostoc citrovorum* 8081, while the term folic acid (FA) has been considered as a generic term applicable to a group of substances, including PGA and CF, which stimulate the growth of *Lactobacillus casei* and *Streptococcus faecalis*.

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TABLE I. Comparison of the Capacity of Various Tissues to Form Citrovorum Factor.

Tissue*	Yield of CF after incubation None	Pteroylglutamic acid 100 μg/vessel	% conversion of PGA to CF
	mμg CF/g of tissue, wet wt		
Liver	273	22400	22.13
Kidney	175	534	.36
Pancreas	25	172	.15
Spleen	8	102	.09
Small intestine	7	75	.07
Brain	4	22	.02
Lung	2	15	.01
Ventricle	2	7	<.01
Pectoral muscle	<1	4	<.01
Blood	<1	<1	0
Bone marrow	3	121	.12

* The tissues, obtained from FA-depleted chicks, were suspended in 5 cc of Krebs-Ringer-phosphate which contained sodium ascorbate (10 mg). The vessels, containing approximately 1 g of tissue slices (blood sample—3 cc/vessel), were incubated at 37° for 1 hr under an atmosphere of nitrogen.

assayed for *Lactobacillus casei* and *Streptococcus faecalis* activity, it was assumed that only 50% of the synthetic vitamin was active for the organism, *Leuconostoc citrovorum;* this assumption has been discussed elsewhere (1). The amounts of CF referred to throughout these studies are calculated on the basis of the naturally-occurring material, which is regarded as exactly twice as active as the synthetic material presently available. For comparison of these with other studies in which the results were related directly to a synthetic reference standard, the amounts of CF reported in such studies must be halved.

Results. The data presented in Table I indicate that the conversion of PGA to CF occurs mainly in the liver. This comparison was consistent with the results of two similar experiments using tissue from chicks. A small amount of CF was formed by all tissues tested (with the exception of blood). Although the assay organism responds slowly when PGA is added to the medium in very large amounts (5 to 10 μg per assay tube), the low value obtained for the sample of blood incubated in the presence of 100 μg of PGA demonstrates that under the conditions of the assay this material was inactive. However, as an additional control, PGA was added to filtrates of the control samples in amounts corresponding to that present in the samples which were incubated with PGA. The control values were not increased by this treatment. Although the amount of CF formed by bone marrow was small, it should be noted that the increase relative to the control value was greater than in any other tissue, with the exception of liver.

Since the concentration of CF in the livers of FA-depleted rats was approximately one-tenth of that which occurred in the livers of normal rats, the increase in the hepatic concentration of CF following administration of folic acid could be clearly demonstrated (Table II). Within one hour following the intraperitoneal injection of PGA, large amounts of CF were present in the livers of the previously depleted rats. Thereafter the concentration of CF decreased, and, after 24 hours, the amount of the vitamin retained in the tissue corresponded closely to that observed in the livers of normal rats.

A comparison of the effect of aerobic and anaerobic conditions on the formation of CF was carried out by incubating slices or homogenates of liver under an atmosphere of nitrogen and, simultaneously, a duplicate series of tissue replicates under oxygen. The results of representative experiments are presented in Table III. The hepatic tissue used in all these experiments was obtained from FA-depleted rats or chicks, a circumstance which minimized the amount of CF released from conjugates of the vitamin during the incuba-

TABLE II. Rate of Appearance of Citrovorum Factor in Hepatic Tissue of Rats Following Intraperitoneal Administration of Pteroylglutamic Acid (PGA).

Time (hr) following I.P. dose of PGA (10 mg/kg)	No. of rats	Citrovorum factor content of liver, μg/g of tissue, wet wt Avg	Range
Folic acid-deficient rats			
Control (untreated)	6	.13	.07– .22
1	4	3.84	2.23–5.12
2	7	2.77	1.53–3.95
4	3	1.99	1.76–2.19
24	6	1.57	.81–2.65
Normal rats			
Control (untreated)	5	1.27	.92–1.47
24	5	2.23	2.14–2.50

TABLE III. Formation of Citrovorum Factor by Liver Preparations Incubated Aerobically and Anaerobically with Pteroylglutamic Acid (PGA).

Additions/vessel*		Yield (μg) of CF/g tissue, wet wt, following incubation under atmosphere of	
PGA, μg	Ascorbic acid, mg	Oxygen	Nitrogen
	Rat liver slices		
0	0	.007	.027
0	10	.032	.055
100	0	.220	1.26
100	5	.920	2.64
100	10	1.51	2.83
100	20	2.10	2.36
	Rat liver homogenate		
0	0	.005	.287
0	20	.015	.514
100	0	.062	2.56
100	10	.253	3.23
100	20	.780	2.51
	Chick liver slices		
0	0	.351	.496
0	10	.592	.596
100	0	3.34	8.80
100	5	11.0	15.2
100	10	12.9	12.9
100	20	15.6	12.7
	Chick liver homogenate		
0	0	.082	.318
0	20	.398	.367
100	0	.470	8.63
100	10	5.73	17.2
100	20	9.52	18.1

* Each vessel contained approximately 1 g of liver slices suspended in 5 cc of Krebs-Ringer-phosphate which contained the additions indicated *or* 4.5 cc of a 1:4 homogenate of liver in neutral phosphate buffer (0.1 M) and 0.5 cc of buffer or the additions indicated. The vessels were incubated at 37° for 1 hr.

tion. Consistently higher yields of CF were obtained under anaerobic conditions. When rat liver slices were incubated in the presence of 100 μg of PGA under an atmosphere of oxygen, the yield of CF was much smaller than that obtained in the duplicate incubated under nitrogen. The addition of sodium ascorbate increased the yield of CF in each case, but 5 mg of ascorbate was more effective under anaerobic conditions than 20 mg under an atmosphere of oxygen. In the experiment using homogenized rat liver, poor yields of CF were obtained in the aerobic incubation, even in the presence of ascorbate, but under anaerobic conditions the formation of CF occurred readily without the addition of ascorbate. In the experiments using chick liver, larger yields of CF were obtained, and a similar favorable effect of anaerobic conditions and the presence of ascorbate was observed. Homogenates of chick liver were capable of forming CF when incubated under oxygen in the presence of ascorbate.

Discussion. The conversion of PGA to CF appears to occur mainly in the liver. The capacity of other tissues to form CF seems to be quite limited. However, it is of interest that bone marrow is able to form an appreciable amount of CF, relative to the control value for this tissue. In cultures of bone marrow obtained from untreated patients with pernicious anemia, Thompson(6) observed that maturation of the megaloblasts was induced by PGA (10 to 50 μg per cc) and Callender and Lajtha(7) found that CF was more effective than PGA in causing the disappearance of megaloblasts in similar cultures. Consequently the ability of bone marrow to form even a small amount of CF may be of real importance with relation to the function of CF in this tissue.

Goldin *et al.*(8) demonstrated that doses of FA administered one hour prior to Aminopterin protected mice against otherwise lethal doses of the antagonist and that this protection did not occur when PGA and the analogue were given at the same time. Data presented here show that PGA was converted rapidly to CF in the liver of intact rats and saturation of the tissue with CF, a substance which can compete effectively with Aminopterin, occurred within one hour following administration of PGA. Although a large dose of PGA (10 mg per kg) was administered, only about 1 μg of CF per g of tissue was retained in the liver at the end of a 24-hour period.

Both anaerobic conditions and the presence of ascorbate facilitated the formation of CF. Studies now in progress indicate that ascorbic acid inhibits the enzymatic destruction of CF during the aerobic incubations. However, since ascorbate increased the yield of CF in the anaerobic medium, it may also participate directly in the formation of CF. The finding that the enzymatic formation of CF proceeds well in cell-free preparations of liver makes possible a detailed study of the properties of the system and the mechanism of the reaction.

Summary. The liver appears to be the main site of the conversion of pteroglyglutamic acid (PGA) to citrovorum factor (CF) in the body. Other tissues had very limited capacity to form CF, with the exception of bone marrow which, relative to the small amount present in this tissue, formed an appreciable quantity of the factor. The hepatic tissue of rats which had been depleted of folic acid and CF was flooded with CF within one hour following the intraperitoneal injection of PGA. Homogenates of rat or chick liver, when incubated with PGA under an atmosphere of nitrogen, were capable of forming CF. Little CF was found when similar preparations were incubated under oxygen. The yield of CF was consistently increased by the presence of ascorbate in the incubation mixture.

The author is grateful to Dr. Arnold D. Welch for his interest in this work. Pteroylglutamic acid and synthetic citrovorum factor (leucovorin) were provided by the Lederle Laboratories Division of American Cyanamid Co., through the courtesy of Dr. T. H. Jukes.

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Hemagglutination with Certain Arthropod-Borne Viruses.* (20299)

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Specific hemagglutination (HA) with neurotropic viruses was first described by Hallauer(1) for the Columbia SK and Columbia MM viruses, by Lahelle and Horsfall(2) for the GD VII virus, and by Olitsky and Yager (3) not only for the Columbia SK and Columbia MM, but also for the Mengo and encephalomyocarditis viruses. Other reports have followed, largely confirming these findings; but departures from them were recorded by Sabin and Buescher(4) and Sabin(5), who included several other encephalitis viruses. The former(4) reported success with Japanese B encephalitis, West Nile disease, and Russian Far Eastern encephalitis viruses; the latter(5) reported success not only with these agents, but in addition with St. Louis encephalitis and Western equine encephalitis viruses.

Recently Sweet, Chanock, and Sabin(6) showed the presence of hemagglutinins associated with 2 immunologically distinct types of dengue virus, Hawaiian and New Guinea C. The method of preparation of viral extracts, the pH range needed for positive reactions, and the conditions under which serum inhibition of HA took place were found to be rigid and exacting and varied from virus to virus. MacDonald(7), in a recent step forward, readily prepared specific HA extracts from the brains of newborn mice infected with Murray Valley encephalitis virus. Prior studies in this laboratory have shown the superiority of the brains of newborn mice as source material for the preparation of complement fixation antigens. It was therefore planned to use such mice[‡] in an attempt to simplify the HA test for certain arthropod-borne viruses. As the following will show, a method was devised which is relatively simple and which gives readily reproducible results.

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The method is essentially that employed for the preparation of complement fixation antigens from mouse brain treated with acetone and ether(8). This antigen as such is the viral extract used in the HA test.

Viruses. The viruses—and their strains—under investigation and the number of passages in mice and other animals for each were: Anopheles A(9): Adult mice, 166; plus newborn mice, 2. Dengue—Hawaiian: Adult mice, 76; plus newborn mice, 4. Dengue—New Guinea B: Adult mice, 43; plus newborn mice, 6. Eastern equine encephalitis: Adult mice, unknown (hundreds); plus newborn mice, 2. Ilhéus (10): Adult mice, 25; plus newborn mice, 2. Japanese B encephalitis—Nakayama: Adult mice, 100 to 200; plus newborn mice, 8. Ntaya (11): Adult mice, 10; plus chick embryos, 7; and newborn mice, 4. Poliomyelitis[§]—Type 2, MEF 1: Adult mice, 20; plus newborn mice, 137. Russian Far Eastern encephalitis—No. 1: Adult mice, unknown number of passages in Russia; adult mice in this laboratory, 10 to 20; plus newborn mice, 1. West Nile—original: Adult mice, 30; plus newborn mice, 1. West Nile—Egypt 101: Chick embryos, 5; plus adult mice, 0; and newborn mice, 5 to 8. Yellow fever—Asibi: Adult mice, 0; plus monkeys, unknown; and newborn mice, 2.

Preparation of brain extracts. Two hundredths ml of the virus, a 10^{-1} suspension of infected brain tissue, was inoculated intracerebrally into 2- to 4-day-old mice. When signs of infection were noticeable, the etherized mice were sacrificed by exsanguination. The brains were frozen rapidly with solid CO_2-alcohol mixture and stored at —25°C until used. Extracts were prepared by treatment of the brains with acetone and ethyl ether as already described(8)—essentially, 2 extractions with acetone, one with a mixture of equal parts of acetone and ether, and 2 with ether. The residue, after drying, was resuspended in 0.9% sodium chloride in distilled water; the suspension represented 1 g tissue in 2 ml diluent. The suspensions were kept overnight at 4°C, then centrifuged at about 9,000 G for one hour. In the end the extract represented a 1:3 dilution of original brain tissue. Dilutions shown in the tables are those of the original brain tissue when introduced into the test tubes. Controls comprised similarly prepared extracts of normal mouse brain. Preparations were stored at —25°C. Repeated freezing and thawing 3 or 4 times apparently did not significantly affect the hemagglutinin titers; 6 or 8 times did, however, cause a definite reduction. One of the preparations (Japanese B encephalitis virus) was lyophilized and this led to a moderate loss of titer.

Erythrocytes. Sheep and human O red blood cells were not generally satisfactory, but cells from one-day-old chicks were employed successfully. They were prepared as described by Sabin(5), *i.e.,* collected in Alsever's solution, and when needed were washed 3 times in physiological saline solution. Of a 0.25% suspension of the washed cells in saline, 0.5 ml was added to each tube in the tests.

Tests. Tubes were kept in an ice bath at 4° to 8°C, the optimal temperature, although tests could also be carried out at room temperature. To each tube was added 0.5 ml of extract in increasing 4-fold dilutions in physiological saline, pH 6.8 or 7, with phosphate buffer, M 0.02. The cells were then added. The tubes were shaken and kept at 4°C for one to 2 hours at which time readings according to Salk's pattern(12) were made. Generally speaking, the lowest dilutions gave a coarse type of agglutination and the moderate and high dilutions gave the shield type.

Results. Two or more different lots of extract of each virus were tested, and each lot was used repeatedly. A summary of the results is given in Table I.

It will be seen that Anopheles A, Eastern equine encephalitis, Type 2 poliomyelitis, Russian Far Eastern encephalitis, and West Nile (original) viruses failed to show agglu-

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[‡] Unless otherwise specified, all brain tissue mentioned hereafter will refer to the brains of newborn mice.

[§] Although not known to be an "arthropod-borne" virus, the poliomyelitis virus is included here because of other properties common to the group.

TABLE I. Hemagglutination with Viruses in Mouse Brain Treated with Acetone and Ether.

Virus	Strain	Titer (expressed as reciprocal of dilution of brain tissue)
Anopheles A		0*
Dengue	Hawaiian	49152
"	New Guinea B	3072
Eastern equine encephalitis		0
Ilhéus		49152
Japanese B encephalitis	Nakayama	196608
Ntaya		3072
Poliomyelitis	Type 2, MEF 1	0
Russian Far Eastern encephalitis		0
West Nile	Original	0
"	Egypt 101	49152
Yellow fever	Asibi	12288
Normal tissue		0

* 0 = dilution <1:12.

tination. It is to be noted that none of the normal brain preparations gave positive results, nor did any positive viral extracts reveal inhibition of hemagglutination in low dilutions. Only when extracts were added undiluted, *i.e.*, dilution of brain tissue 1:3, was there often inhibition, but this was not evident in the lowest dilution employed in the tests, 1:12.

Specific inhibition. In order to establish the specificity of the reactions, known immune sera were tested for inhibition against several of the viruses under study. Prior to their use in the tests, the sera were treated in the manner described by Sabin and Buescher(4) and Sabin(5) in order to eliminate nonspecific inhibitors. Thus far, only monkey and mouse sera have been used; studies are currently being carried out with human sera. The animal sera were prepared as follows: They were heated at 56°C for 30 minutes, then diluted 1:10 in physiological saline. Twelve volumes of acetone were added. After low-speed centrifugation, the sediment was re-extracted with 12 volumes of acetone, centrifuged, dried, and resuspended in physiological saline in a proportion to yield a 1:10 dilution of serum. The HA inhibition tests were carried out with serial 2-fold dilutions of serum in saline containing phosphate buffer M 0.02 at pH 6.8; the first dilution of serum was 1:10 and the volume was 0.25 ml. The tubes containing the serum dilutions were then placed in an ice water bath at 4° to 8°C and the antigen added. This consisted of 0.25 ml of the brain extract diluted in such a way as to provide 16 hemagglutinating units in that volume. Finally, 0.5 ml of the 0.25% suspension of chick erythrocytes was added, and the tubes were kept in the cold room at 4°C.

Table II shows the results of tests with

TABLE II. Inhibition of Hemagglutination with 16 Agglutinating Units.

Virus	Serum	Serum dilutions							
		10	20	40	80	160	320	640	1280
West Nile, Egypt 101 strain	Monkey 49-60, pre-infection	+	+	+	+	+	+	+	+
	convalescent	0	0	0	0	0	±	+	+
	Monkey 49-69, pre-infection	+	+	+	+	+	+	+	+
	convalescent	0	0	0	0	±	+	+	+
Dengue, Hawaiian strain	Monkey 49-75, pre-infection	+	+	+	+	+	+	+	+
	convalescent	0	0	0	0	0	0	±	+
	Mouse, normal	±	+	+	+	+	+	+	+
	" hyperimmune	0	0	0	0	0	0	0	+
Japanese B encephalitis	Monkey 49-35, pre-immunization	+	+	+	+	+	+	+	+
	hyperimmune	0	0	0	±	+	+	+	+
	Mouse, normal	+	+	+	+	+	+	+	+
	" JBE, hyperimmune	0	0	0	0	0	±	+	+
	" EEE "	+	+	+	+	+	+	+	+

+ = agglutination; 0 = no agglutination; ± = questionable agglutination.

West Nile (Egypt 101 strain), dengue (Hawaiian strain), and Japanese B encephalitis viruses. The sera from monkeys infected or hyperimmunized with the respective viruses inhibited HA, while the control samples of serum taken prior to infection or immunization failed to do so. Furthermore, it can be seen that 2 different pools of normal mouse serum did not affect HA with dengue (Hawaiian strain) or Japanese B encephalitis viruses; nor did an Eastern equine encephalitis hyperimmune mouse serum inhibit HA with Japanese B encephalitis virus. The hyperimmune homotypic mouse sera, however, inhibited agglutination in dilutions from 1:160 (for Japanese B encephalitis) to 1:640 (for dengue, Hawaiian strain).

Summary. A simple, easily reproducible method for preparation of high-titer hemagglutinins with several arthropod-borne viruses is described. The method is based on the use of brains from newborn mice infected with virus as the source material, and on treatment with acetone and ether for the elimination of nonspecific factors. With this method, stable specific HA has been found associated with the following viruses: dengue (Hawaiian type), dengue (New Guinea B type), Ilhéus, Japanese B encephalitis, Ntaya, West Nile (Egypt 101 strain), and yellow fever (Asibi strain). As yet, no agglutination has been uncovered associated with Anopheles A, Eastern equine encephalitis, poliomyelitis (Type 2, MEF 1 strain), Russia Far Eastern encephalitis, and West Nile (original strain) viruses.

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Failure of Rutin and Catechin* to Affect Vascular Lesions of Experimental Hypertension or Hypersensitivity.† (20300)

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In a previous communication, one of the authors, (J.L.O.), in collaboration with several others, reported the effectiveness of rutin in decreasing the gross hemorrhage in experimental malignant hypertension in dogs(1). Shortly after that work was completed, Moss, Beiler and Martin(2) reported the effectiveness of another flavonol, catechin, in preventing anaphylactic shock in guinea pigs. Because we were interested in both hypersensitivity arteritis and malignant hypertension, it was decided to test the effectiveness of each of these two compounds in hypersensitivity arteritis and also in the arteritis of malignant hypertension.

Methods. Seven rabbits and 6 dogs were given a solution of rutin in 0.1 N NaOH with

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* Catechin was generously supplied by the National Drug Co., Philadelphia, Pa.

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0.5 cc of 1% procaine subcutaneously, at the rate of 20 mg/kg/day. These injections were given to the rabbits for 12 days before the first injections of horse serum, and to the dogs for from 15-20 days before the stenosis of the renal arteries. In both groups the treatment was continued until the animals died or were sacrificed. This schedule of injections was used in all of the following groups. Seven rabbits and 5 dogs were given a solution of catechin in 0.1 N NaOH with 0.5 cc of 1% procaine subcutaneously. The dogs were given only 8 mg/kg/day, the dosage used by Moss, Beiler and Martin in guinea pigs, but it was decided to give the rabbits the catechin in the same dosage as the rutin, that is, 20 mg/kg/day. The third group of 7 rabbits and 5 dogs were given only 0.5 cc of 1% procaine subcutaneously per day and served as the control group. The rabbits were sensitized by a single intravenous injection of 10 cc/kg of sterile horse serum, and were sacrificed 14 days later. In preparing dogs for the experiment, control blood pressures were measured 3 times per week by a direct femoral puncture until mean blood pressures were consistently below 135 mm Hg for at least 2 weeks. Malignant hypertension was then produced by stenosis of both renal arteries. The stenosis was produced by including a stilet 0.5 mm in diameter within a silk ligature around the artery, the stilet then being withdrawn. After stenosis of the renal arteries, blood pressures were taken daily. All animals were autopsied, and the heart, aorta, lungs, liver, gall-bladder, spleen, pancreas, adrenals, kidneys, urinary bladder, stomach, intestine, colon and mesentery examined both macroscopically and microscopically. The blocks for microscopic examination were taken in the same manner as described previously(3). An arbitrary system was established for the rough quantitative grading of vascular disease in these animals. Since the rabbits had no grossly visible lesions, only grading of microscopic lesions could be made. The lesions in each organ were graded from 0 to 3+ and the total number of plus signs was determined for each animal. A scale for comparison was then made by dividing the total number of plus signs for the animal having the most lesions into 4 equal parts. The scale was then ½ to 3 = +; 3½ to 6 = 2+; 6½ to 9 = 3+; 9½ to 12 = 4+. The use of a final 4+ scale for the rabbits was decided upon since one rabbit had much more extensive lesions than any of the others. This fact could be appreciated in the chart only when a 4+ scale was used. The results for the dogs were similarly computed, except that a final 3+ scale was used.

TABLE I. Degree of Vascular Damage Induced by Horse Serum in Treated Rabbits. 7 animals in each series.

Type of pretreatment	No. of animals with degree of arteritis				
	0	+	++	+++	++++
Control (procaine only)	4	1	2	0	0
Catechin and procaine	4	1	2	0	0
Rutin and procaine	3	0	1	2	1

Results. As shown in Table I, rabbits made hypersensitive to a large intravenous dose of horse serum varied greatly in the amount of vascular damage induced. In spite of this variation, the catechin-treated group and the control group showed essentially the same results. The rutin-treated group, however, developed even greater vascular damage than the control group.

The changes in the dogs could be seen grossly as hemorrhages and microscopically as acute necrosis of arterioles and myocardial necrosis. Accordingly, a rough quantitation of both the macroscopic and microscopic changes was made. As in the rabbits with hypersensitivity, neither rutin nor catechin had any effect on the lesions of dogs with malignant hypertension. These results are presented in Tables II and III.

The results reveal no difference in incidence of lesions between the control and flavonol-treated animals. The only apparent variation is in the rutin-treated group of dogs. Two dogs in this group were completely free of lesions. However, these two animals, in contrast to all of the others, did not develop hypertension after renal artery stenosis and are, therefore, considered to be failures in experimental technic. When this factor is con-

TABLE II. Degree of Hemorrhage Seen Grossly in Treated Dogs with Malignant Hypertension.

Type of pretreatment	No. of animals	No. of animals with each degree of hemorrhage			
		0	+	++	+++
Control (procaine only)	5	1	3	1	0
Catechin and procaine	5	1	3	1	0
Rutin and procaine	6	2	1	2	1

TABLE III. Arteriolar Necrosis and Myocardial Necrosis in Treated Dogs with Malignant Hypertension.

Type of pretreatment	No. of animals	No. of animals with each degree of arteriolar necrosis			
		0	+	++	+++
Control (procaine only)	5	0	3	2	0
Catechin and procaine	5	0	2	3	0
Rutin and procaine	6	2	0	4	0

sidered, the apparent difference in the rutin-treated group loses its significance.

Discussion. Two reviews of the literature pertaining to the flavonol compounds indicate wide variations in the effectiveness of these substances in hemorrhagic states and other vascular disease(4,5). Contrary to the previous observations of one of us (J.L.O.), the results obtained in this experiment fail to indicate any effectiveness of rutin in malignant hypertension in dogs. The only differences between this experiment and the previous one using rutin are the use of different samples of rutin and a different injection schedule. The injection schedule used previously was three times daily, whereas in this experiment, the injections were given once daily. It is possible that either or both of these factors may have caused the difference in results.

Rutin was also found to be ineffective in the prevention of arterial lesions in hypersensitivity. Catechin was ineffective in both types of vascular lesions. It would appear, therefore, that if either flavonol has any effect, it must be too slight to be demonstrated by the procedures used.

Summary. Neither rutin nor catechin altered the vascular lesions of hypersensitivity arteritis in rabbits or of experimental malignant hypertension in dogs.

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Myotrophic Activity of 19-Nortestosterone and Other Steroids Determined by Modified Levator Ani Muscle Method.* (20301)

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Many steroids produce both androgenic and protein anabolic effects(1). Protein anabolic effects have been measured by body weight gain(2), by nitrogen retention(3-6), and by renotropic activity(7,8). Eisenberg and Gordon(9) have proposed a myotrophic test for the assay of protein anabolic activities of androgens. Steroids which had the greatest protein anabolic activity as measured by the classical nitrogen retention methods, also had the greatest myotrophic activity as measured by a levator ani muscle assay(9,10).

In this study a modification of the Eisenberg and Gordon myotrophic assay is proposed which eliminates the 23-day post-castration

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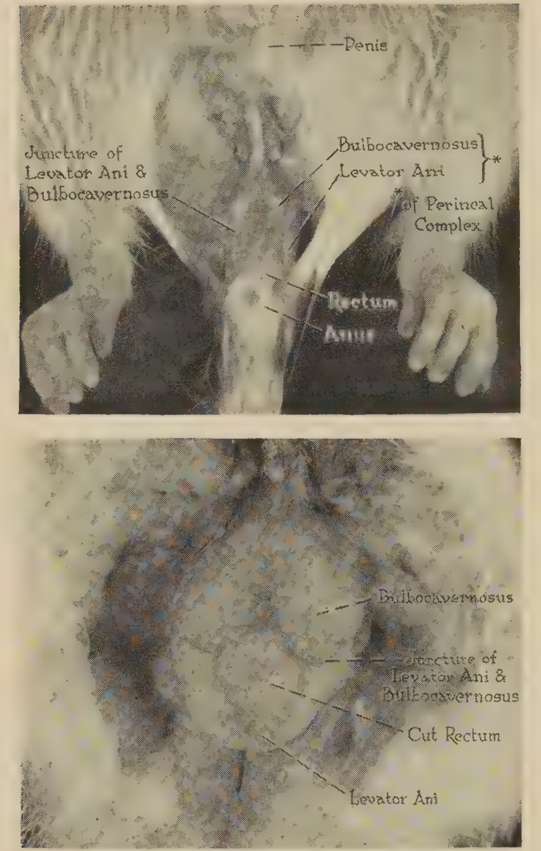

FIG. 1 and 2. Levator anti muscle and related structures in the rat.

rest period for the animals and reduces total assay time from 31 days to 8 days. The advantages are: 1) the rapid turnover of animals reduces the required size of the assay colony, and 2) it becomes possible to start an assay at any time, without the long post-castration delay. The assay has been used in this laboratory to study a number of steroids not previously tested in order to establish a ratio between their myotrophic and their androgenic activities. A series of compounds possessing high myotrophic activity with little or no androgenic activity might be of practical value, as well as of theoretical interest.

Materials and methods. Male rats of the Holtzman-Rolfsmeyer strain, 21 days of age, were castrated and maintained on Rockland Farm rat diet and tap water throughout the experiment. Each animal was given daily subcutaneous oil injections of the test substances for 7 days, beginning with the day of castration. On the 8th day, 22-26 hours after the last injection, the animals were sacrificed. Dissection of the levator ani muscle was effected after removing the skin in the scrotal area between the base of the penis and the anus. The posterior aspect of the perineal complex (Fig. 1) was cleared of fat and connective tissue with forceps, particular care being taken to disclose the constrictions at either end of the levator ani where it joins the bulbocavernosus muscle. The rectum was transected just caudad of the point where the levator ani loops around it dorsally (Fig. 2). The body of the levator ani was then freed of the rectum and removed by incisions at the points of attachment to the bulbocavernosus muscle. The levator ani was cleared of any connective tissue and weighed to the nearest 0.1 mg on a torsion balance. The ventral prostate and seminal vesicle weights (free of coagulating glands) were also recorded, for comparison of androgenic and myotrophic

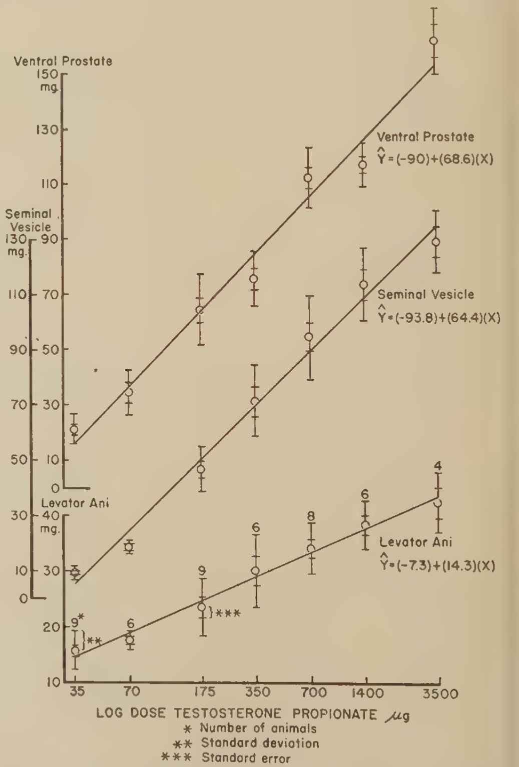

FIG. 3. Responses of the levator ani, ventral prostate, and seminal vesicles to increasing doses of testosterone propionate.

TABLE I. Myotrophic and Androgenic Activities of Standard Steroids and Some of Their Analogues.

Treatment		Total* dose, mg	Body wt, mg	Ventral prostate wt, mg	Seminal vesicle wt, mg	Levator ani wt, mg	Lev. ani /v. pros. ratio†
Castrated control	(25)‡	—	65	9.7± .4	7.0± .3§	12.2± .5	
Testosterone propionate	(9)	.035	68	21.5±1.9	9.8± .4	15.7±1.1	.30
	(6)	.070	67	34.8±3.5	18.7±1.0	17.6± .7	.22
	(9)	.175	68	64.7±4.3	47.0±2.8	23.7±1.7	.21
	(6)	.350	68	75.8±4.0	71.7±5.1	30.1±2.7	.27
	(8)	.700	67	112.1±3.9	94.5±5.4	34.2±1.6	.21
	(6)	1.400	69	114.7±3.1	113.7±5.3	38.4±1.7	.25
	(4)	3.500	71	161.7±6.0	128.8±5.6	42.3±2.7	.20
Testosterone	(6)	.350	62	35.3±1.8	14.7± .9	20.4±2.3	.32
	(8)	.700	68	46.5±4.6	17.8±1.3	21.9±2.0	.26
	(11)	1.400	66	48.4±2.3	27.4±2.3	23.7±1.0	.30
	(8)	2.100	70	63.5±4.5	38.2±5.5	28.8± .9	.31
	(9)	3.500	68	71.3±4.9	51.6±4.4	28.7±2.2	.27
[a]19-Nortestosterone	(5)	.700	66	19.7±2.5	12.1±1.7	21.6± .5	.94
	(11)	1.400	73	20.7±1.8	12.5± .6	24.5±2.0	1.10
	(5)	2.100	70	27.5±5.1	14.4±1.1	28.1±2.2	.89
	(6)	3.500	67	21.0±2.2	15.4±1.1	26.0± .6	1.20
[a]17-β Hydroxy-5(10) estren-3-one	(6)	2.800	66	12.7± .7	15.1±1.1	20.0±2.2	2.60
	(6)	3.500	63	14.1± .5	15.9±1.2	18.7±1.0	1.50
[a]19-Norandrostenedione	(6)	3.500	68	22.4±1.4	9.7± .7	18.3± .7	.48
Methylandrostenediol	(5)	.140	64	16.2±1.1	7.3± .3	11.4±1.7	
	(6)	.350	66	24.0±1.8	9.0±1.0	14.4±1.9	
	(11)	.700	67	29.5±1.7	11.6± .6	15.4± .9	.16
	(5)	1.400	69	37.1±2.3	16.5±1.3	17.0±1.0	.18
	(5)	1.750	65	55.3±2.9	33.6±2.7	22.9±1.1	.23
	(3)	3.500	65	66.3±5.3	58.5±6.2	25.8±1.1	.24
[b]Androsterone	(3)	.672	69	69.9±8.9	7.6± .5	15.5± .5	
	(6)	1.750	68	93.0±3.7	11.2± .5	16.6±1.3	.05
	(6)	3.500	64	109.4±4.1	13.8± .7	15.4±2.0	
Estrone	(4)	21.000	51	10.1±1.0	21.0± .8	8.8±1.0	
Estradiol dipropionate	(4)	7.000	54	9.3± .6	18.8± .5	10.0± .5	
[c]Cortisone acetate	(4)	21.000	55	9.4± .8	6.5± .7	6.6± .3	
Desoxycorticosterone-acetate	(6)	7.000	71	9.6± .8	7.7± .4	11.5± .7	
Progesterone	(6)	3.500	68	29.5±2.8	7.4± .8	13.4±1.2	
[d]19-Norprogesterone	(5)	3.500	75	14.1± .8	7.1± .9	14.7±1.2	
Pregnenolone acetate	(6)	3.500	66	17.3±3.0	7.1± .2	14.3± .9	

* Administered subcut. once/day for 7 days.

† Ratio of increase in levator ani to increase in ventral prostate. (Included only if levator ani response showed a highly significant increase, $P < .01$, over controls.)

‡ Figures in parentheses represent No. of animals used.

§ Stand. error.

Furnished by: [a] Drs. A. L. Wilds and N. A. Nelson, Univ. of Wis.; [b] Ciba Pharmaceutical Products; [c] Merck and Co.; [d] Chemical Specialties, Inc.

activity. Dry weights were taken on the levator ani muscles following the method of Astwood(11), except that the muscles were desiccated at 72°C.

Results and discussion. Testosterone propionate was selected for a dose-response curve (Fig. 3), since it has been shown to be highly active in producing the classical androgenic stimulation of the accessory organs of reproduction, in nitrogen retention(12), and, more recently, it has been shown to possess high myotrophic activity(9). In this study responses to testosterone propionate by the levator ani muscle and by the accessory glands of reproduction are termed the myotrophic and the androgenic activities, respectively.

When the weights obtained in these experiments for the levator ani, ventral prostate and seminal vesicles are plotted against the log of the dose of testosterone propionate, a straight line is obtained for each end organ. The slope of the levator ani dose-response curve does not parallel those for the accessory glands; the curves for ventral prostate and seminal vesicles are approximately parallel (Fig. 3). The organ weight responses obtained in these experiments (Table I) are in agreement with previous observations of Eisenberg and Gordon(9) that testosterone propionate is more active, both androgenically and myotrophically, per unit weight than other commonly used androgens. However, in evaluating compounds for possible myotrophic use where androgenic activity is undesirable, the ratio of the response of the levator ani to the response of the ventral prostate may be an important consideration. In order to compare the myotrophic activity of test substances to their classical androgenic activity, the ratios of the levator ani response to the ventral prostate response (myotrophic/androgenic ratios) were devised. The levator ani/ventral prostate ratios (l.a./v.p. ratios) in Tables I and II were calculated as follows:

$$\frac{\text{l.a.}}{\text{v.p.}}\text{ ratio} = \frac{\text{experimental levator ani wt} - \text{control l. ani wt}}{\text{experimental v. prostate wt} - \text{control v. prost. wt}} = \frac{\text{increase in levator ani wt}}{\text{increase in v. prostate wt}}$$

Using the responses to a dose of 35 μg testosterone propionate (Table I) as an example,

TABLE II. Steroids with Weak Myotrophic and Androgenic Activity.

		Total* dose, mg	Body wt, g	Ventral prostate wt, mg	Seminal vesicle wt, mg	Levator ani wt, mg	Lev. ani /v. pros. ratio†
Castrated control	(25)‡	—	65	9.7	7.0	12.2	
[a]Allopregnane-3(β), 17(α)-diol-20-one	(3)	1.40	60	11.9	9.5	13.8	
[b]10-Methyl-6-(trans-4′-hydroxy-cyclohexyl)-$\Delta^{1:9}$-octalone-2	(6)	7.00	66	8.9	6.8	14.2	
[b]6-(4′-Ketocyclohexyl)-Δ^{1-9}-octalone-2	(3)	7.00	60	9.4	7.3	14.8	
[b]6-(Cis-4′-hydroxycyclohexyl)-Δ^{1-9}-octalone-2	(3)	7.00	74	14.6	7.7	15.9	
[a]$\Delta^{1,4}$-Androstadiene 3,17-dione	(5)	1.40	62	27.3	7.1	11.7	
[a]$\Delta^{4,6}$-Androstadiene-dione 3,17	(3)	.70	64	21.4	9.2	13.0	
[a]2-Acetoxy-testosterone-acetate	(3)	.17	62	39.4	9.4	14.1	
[c]1,8-Diketoperhydro-10a-methyl chrysene	(3)	2.55	71	10.7	7.9	15.0	
[a]$\Delta^{1,4,6}$-Androstatriene-17-ol-3-one-acetate	(2)	1.05	68	16.5	10.5	15.5	
[d]17-Vinyl-Δ^{4}-androsten-3-one-17(β)ol	(3)	1.40	64	42.6	22.7	18.5	.19
[a]16-Methyl-17-iso-Δ^{4}-pregnene-3,20-dione	(3)	3.50	66	15.1	6.5	12.8	
[d]Dehydroisoandrosterone	(4)	4.20	74	35.5	8.4	13.6	
[a]Δ^{1}-Androstenedione-3,17	(3)	3.50	56	12.9	8.2	15.2	
[a]Androstane-3,17-dione	(3)	3.50	69	122.3	12.3	18.0	.05
[a]Δ^{4}-Androstene-17(α)-ol-3-one-acetate	(3)	3.50	63	33.2	11.4	15.0	
[a]$\Delta^{3,5}$-Androstadiene-3,17-dione-3-enol ethyl ether	(3)	5.25	68	56.1	10.3	15.1	
[a]$\Delta^{5,16}$-Pregnadiene-3(β)-ol-20-one acetate	(3)	3.50	69	10.7	7.5	12.0	
[a]Allopregnane 17(α)-ol-3,20-dione	(3)	3.50	72	43.2	8.3	14.7	
[a]Allopregnane-3(β), 17(α), 21-triol-20-one-3,21-diacetate	(3)	3.50	68	11.0	8.6	14.8	
[d]17-Iso-5-pregnene-3 β, 17 β-diol	(3)	1.75	64	15.4	9.4	15.2	

* Administered subcut. once/day for 7 days.

† Ratio of increase in levator ani to increase in ventral prostate. (Included only if levator ani response showed a highly significant increase, $P < .01$, over controls.)

‡ Figures in parentheses represent No. of animals used.

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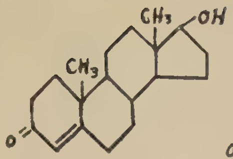
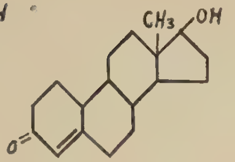
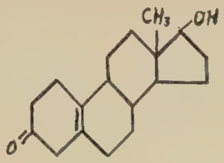
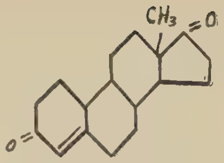

Testosterone


19-Nortestosterone


17 β -Hydroxy-5(10)-estren-3-one


19-Norandrostenedione

the experimental levator ani weight of 15.7 mg minus the castrated control weight of 12.2 mg gave an *increase* in muscle weight of 3.5 mg. Likewise, the experimental ventral prostate response of 21.5 mg minus the control weight of 9.7 mg gave an *increase* of 11.8 mg for the prostate. The ratio of these two increases, 3.5/11.8 = 0.30, is the l.a./v.p. ratio. The l.a./v.p. ratios for testosterone propionate range between 0.20 and 0.30 at all doses studied. Using this method of comparison any relative increase in levator ani response would be reflected in an increase in the l.a./v.p. ratio.

Since these assays were primarily intended for assessing myotrophic activity, the myotrophic/androgenic ratios in Tables I and II were calculated for only those dose-levels which produced a highly significant ($P < 0.01$) increase in levator ani weight. Levator ani/seminal vesicle ratios were not calculated because the seminal vesicles are less sensitive than the ventral prostate to small quantities of most androgens and to all dose levels of some well known androgens, *e.g.* androsterone(13). Furthermore, the seminal vesicle weights are believed to be affected more by non-androgenic steroids(14). Although steroids are often known to produce hydration of the tissues, there was no evidence of hydration of the levator ani muscles in this experiment as shown by the dry weight determinations. Therefore all myotrophic/androgenic ratios are taken directly from the wet weights of the organs.

Using this method, l.a./v.p. ratios were calculated for testosterone, testosterone propionate, and methylandrostenediol, which have been recognized as myotrophic agents. The l.a./v.p. ratios found for methylandrostenediol were of the same order of magnitude as those found for testosterone propionate. This does not support the reports that methylandrostenediol is more valuable than testosterone propionate as a protein-anabolic compound (10,15).

A group of compounds lacking the C-19 methyl group was of special interest to us (16). The group included 19-nortestosterone, 17 β-hydroxy-5(10)-estren-3-one, and 19-norandrostenedione, all of which are analogues of familiar androgens. It can be seen from Table I that 19-nortestosterone, 17 β-hydroxy-5(10)-estren-3-one, and 19-norandrostenedione were compounds which showed l.a./v.p. ratios greater than those for testosterone propionate and for unesterified testosterone. The l.a./v.p. ratios for testosterone propionate and free testosterone were very similar, while the ratios for 19-nortestosterone were much higher. 17 β-hydroxy-5(10)-estren-3-one (a double-bond isomer of 19-nortestosterone) showed even higher l.a./v.p. ratios, while 19-norandrostenedione, at the single dose level given, showed a ratio which was not as striking as for the other 19-nor compounds but was greater than for the other standard androgens tested. It is evident that a minimum of androgenic activity is coupled with definite myotrophic activity in the 19-nor compounds.

Since the preparations of 19-nortestosterone and its isomers were not esterified the responses to these compounds should be compared to those for free testosterone rather than testosterone propionate. Equal doses by weight of free testosterone and 19-nortestosterone produced approximately equal myotrophic responses. An examination of the androgenic activity in response to equal doses of the 2 compounds reveals, however, that the increases in ventral prostate weights for testosterone treated groups are much greater than the increases following 19-nortestosterone administration. Thus, while the *myotrophic* activities of the 2 compounds are approximately equal, the *androgenic response* to 19-nortestosterone is markedly lower than the androgenic response to testosterone. Chick comb assays obtained in this laboratory (unpublished) indicate that testosterone is approximately 4 times as active androgenically as 19-nortestosterone, while the ventral prostate responses in the rat show testosterone to be about 10 times as active androgenically in this species.

The myotrophic activity of these preparations suggests that the muscle response is limited to androgens and to analogs of androgenically active compounds, which may possess a minimum of androgenic activity *per se.* A number of hormonally active non-androgenic compounds which do not elicit the myotrophic response have been included in Table I for comparison. Progesterone, 19-norprogesterone, pregnenolone acetate, estrone, estradiol dipropionate, cortisone acetate, and desoxycorticosterone acetate were shown to have no significant stimulatory effect on the levator ani muscle when the total doses indicated in Table I were administered. Additional evidence for the specificity of the myotrophic response to androgens and their isomers is found in Table II where the results of tests on a number of other compounds are tabulated. Only 2 compounds in Table II (17-vinyl Δ^4-androsten-3-one-17 β-ol and androstane-3,17-dione) showed myotrophic activity ($P < 0.01$) and in each case androgenic activity was sufficient to produce a l.a./v.p. ratio similar to the testosterone ratios.

Although to date steroidal myotrophic activity has been found associated with androgens or analogs of androgens, there is a distinct lack of parallelism between myotrophic and androgenic activities. This finding is in agreement with the data of Eisenberg and Gordon(9). The 19-nor analogs which have been tested (Table I) show high myotrophic/androgenic ratios while, on the other extreme, androsterone (Table I) and androstane-3,17-dione (Table II) exhibit very low myotrophic/androgenic ratios. The favorable myotrophic/androgenic ratios shown for the 19-nor compounds tested would suggest that the 19-nor series of steroids may be valuable for their protein-anabolic activity.

Summary. An 8-day rat levator ani muscle assay for myotrophic activity is presented. Preliminary steroid screening results indicate that 19-nortestosterone and other 19-nor analogs of androgens promise to be effective protein-anabolic, and relatively weak androgenic agents. Using the same dose levels of 19-nortestosterone and testosterone, equal myotrophic responses were produced, yet 19-nortestosterone showed only weak androgenic activity and testosterone exhibited strong androgenic activity. Androsterone possesses relatively strong androgenic and only weak myotrophic activity.

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Influence of Cortisone on Thyroid-Pituitary Interaction in Normal and Goitrous Rats.* (20302)

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It is difficult to reconcile the conflicting reports regarding the effect of cortisone on the pituitary-thyroid system. In man, prolonged administration of cortisone appears to inhibit thyroid function as measured by thyroidal I^{131} uptake and serum protein-bound iodine (1-3) but other indices of thyroid function (BMR, serum cholesterol) respond less consistently(1,3,4). It has been postulated that this thyroid inhibition—and its sequel, "corticogenic hypothyroidism"—is a consequence of pituitary TSH suppression(1,4). Results of animal experimentation do not fully support this. In the intact rat, cortisone administration may(5,6) or may not(7) reduce thyroidal I^{131} uptake, and is without effect on the rate of release of labeled thyroid hormone(6,8). The histologic response of the thyroid to cortisone is variable; diminished activity(9), no change(10), and activation (11,12) have been reported. In the hypophysectomized rat, simultaneously administered cortisone and TSH inhibits TSH-induced thyroidal radioiodine uptake(13-15), but fails to prevent the expected increase in thyroid cell height(16). In no instance have the levels of TSH in blood and pituitary in cortisone-treated animals been measured and correlated with other parameters of thyroid-hypophyseal interplay. The present study supplies this information for both normal and hypothyroid rats.

Materials and methods. Cortisone[¶] was administered (dosage and duration in Table I) to adult female rats (Albino Farms, Red Bank, N. J.) by subcutaneous injection. All rats receiving cortisone were fed unrestricted amounts of the normal ration (Purina chow and tap water). For control groups, food was limited to amounts consumed by experimental groups for the previous 24-hour period (pair-fed) or was given *ad libitum*. In the first goitrogen experiment, cortisone and propylthiouracil[‖] (PTU; 0.05% in the ration) were given concurrently throughout (18 days); in the second experiment, the goitrogen was given for 130 days, cortisone being administered for the last 24 days only. The thyroid, hypophysis, adrenals, and thymus were quickly removed at sacrifice (20-24 hours after last injection), were trimmed, weighed on a microtorsion balance, and prepared for histologic study. In the radioiodine studies, 4 μc of I^{131} (carrier free)[**] were injected intraperitoneally 24 hours prior to sacrifice. Radioactivity in single thyroid lobes was determined by a method described previously(17). Extracts of adenohypophyses for TSH assay were made by a standardized procedure(18). The pituitary extracts, and also the serum obtained from pooled blood of animals in respective groups were bioassayed for TSH in the stasis tadpole(19).

Results. The results are summarized in Table I. Cortisone invariably caused body weight loss. Cortisone treatment alone produced no significant change in thyroid- or adenohypophyseal-body weight ratios and was without significant effect on the TSH content of the pituitary. Blood TSH titers in cortisone-treated animals and in pair-fed controls were diminished as compared with those in normally fed rats. Adrenal atrophy and thymic involution were found consistently in all cortisone-treated animals. The smallest ad-

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[¶] Cortisone was obtained from Merck and Co. through the courtesy of Dr. E. Alpert.

[‖] Propylthiouracil was supplied by the American Cyanamid Co. through the courtesy of Dr. S. M. Hardy.

[**] I^{131} was obtained from the Atomic Energy Com., Oak Ridge.

TABLE I. Effect of Cortisone on TSH Content of Blood and Adenohypophysis.

Group	No. of animals	Mean body wt (g) Initial	Mean body wt (g) Final	Mean gland wt (mg) 100 g final body wt Adenohyp.	Mean gland wt (mg) 100 g final body wt Thyroid	% I131 uptake Per mg thyroid	% I131 uptake Total gland	TSH assay in Stasis tadpole Serum† Thyr. cell ht.	TSH assay in Stasis tadpole Serum† TSH/μg/ml‡	TSH assay in Stasis tadpole Adenohypophysis† Thyr. cell ht.	TSH assay in Stasis tadpole Adenohypophysis† TSH/μg/mg‡
Normal, fed *ad lib.*	10		204±15*	4.5±.3	7.0± .4	—	—	5.9±.15	.36(.32– .44)	9.3±.24	21.7(15.0–26.7)
Cortisone, 2.5 mg daily for 15 days	10	196±12	180±17	5.3±.4	7.7± .3	—	—	5.4±.14	<.20	8.9±.35	13.3(10.0–20.0)
Normal, pair-fed	10	187±13	190±13	4.7±.4	8.4± .4	—	—	5.2±.05	<.20	9.2±.21	20.0(15.0–23.3)
PTU and cortisone, 2.5 mg daily, 18 days	12	226±23	195±22	5.3±.3	26.6±1.4	.026±.005	1.4± .9	6.7±.07	1.00(.88–1.08)	8.0±.10	4.3(4.0– 5.3)
PTU, pair-fed	12	225±15	213±15	4.6±.3	19.8±1.4	.030±.004	1.4± .2	6.2±.14	.60(.44– .64)	7.9±.33	4.0(2.7– 6.0)
PTU, 130 days, and cortisone, 2.5 mg daily, 24 days	9	163±14	125±12	9.3±.8	112.0±8.2	.032±.004	4.5± .6	6.9±.15	1.18(.90–1.33)	6.7±.20	.8(.7– 1.1)
PTU, 130 days	8	170±15	151±13	6.4±.5	54.0±4.1	.040±.005	3.2± .4	6.8±.17	1.08(.88–1.26)	7.3±.20	1.6(1.3– 2.3)
Normal, fed *ad lib.*	9		202±15	3.2±.3	6.6± .3	.575±.080	7.6±1.1	5.8±.07	.30(.26– .36)	8.8±.27	12.3(9.0–16.7)

* Mean body wt ± avg deviation; in all other columns ± values refer to stand. error of mean.

† Test animals received 0.05 ml of serum daily, 5 successive days; total amt of pit. extract given (over 4 inj.) represented .30 mg adenohypophysis.

‡ TSH concentration given in mean μg equivalents (range) of a standardized TSH preparation (D'Angelo, *Endocrinology*, 1951, v48, 249).

renals occurred in rats receiving both cortisone and PTU.

The simultaneous administration of cortisone and PTU induced marked changes in thyroid and pituitary. In rats receiving both cortisone and goitrogen for 18 days, thyroid hyperplasia was greater (34%) than with the goitrogen alone. Thyroid size in the latter group was approximately 3 times that of normal controls. Thyroid hyperplasia was accompanied by increased concentration of serum TSH. Hormone titers were higher in animals receiving cortisone and PTU than in those treated with PTU alone. TSH concentrations in the pituitary were substantially and equally reduced after 18 days of goitrogen treatment with or without cortisone.

The effect of cortisone on the thyroid and adenohypophysis of goitrous rats was found to be more marked if goitrogen treatment was prolonged prior to administration of the steroid. Under these circumstances, thyroid-body weight ratios in cortisone-treated rats were twice greater than with goitrogen alone and were increased to approximately 1700% of normal. In contradistinction to the findings in the short term experiment, pituitary enlargement occurred after 130 days of ingestion of PTU. This was significantly greater in the cortisone-PTU-treated animals. Pituitary TSH concentrations in goitrous animals were reduced still further, with lowest values found in the cortisone-PTU group. Diminished TSH stores were only partially offset by the increase in pituitary size. Blood TSH concentrations, however, were elevated equally in both groups.

As expected, there was marked diminution in the thyroidal I^{131} uptake of PTU-treated rats. The decreased avidity of thyroid tissue for radioiodine was not significantly modified by cortisone administration. Total radioiodine accumulations were below normal although the increased thyroid mass in cortisone-treated animals (2nd experiment) resulted in significantly greater I^{131} collections than with goitrogen alone.

Discussion. It is evident that the level of thyroid function may itself condition the response of the pituitary-thyroid system to high levels of circulating adrenocortical steroids. Cortisone increases the size of the thyroid and adenohypophysis under conditions of inhibited thyroid hormone formation. Expected changes in blood and pituitary TSH levels are also induced in goitrous rats with cortisone. However, our data on TSH levels do not establish a strict parallelism as regards time sequence. The absence of such responses in the normal animal indicates that this steroid is not itself a goitrogenic agent. The decrease in blood TSH levels found in both cortisone-treated and pair-fed rats may well result from the reduced food intake. It is known that in acute starvation blood TSH levels fall(20). Lowering of the blood TSH titers due to malnutrition could conceivably account for some of the reduction in thyroidal radioiodine uptake occasionally reported in the rat.

The claim by Lederer(21) that cortisone depresses thyroid function through inhibition of TSH secretion from the pituitary was not confirmed. The decrease in TSH content found by Lederer in the hypophyses of thyroidectomized rats given cortisone may have resulted from augmented release; blood TSH levels were not measured. The present study reveals that enhancement of pituitary TSH secretion with cortisone occurs in the hypothyroid but not in the euthyroid animal. Halmi and Barker(12) have described morphologic changes in thyroid and hypophysis which suggest that cortisone promotes TSH function of the pituitary in non-goitrous rats. Differences in diet, or in dosage and duration of hormone treatment may possibly account for the dissimilar findings. The potentiating action of cortisone on PTU-induced goitrogenesis may be mediated through a direct action of the steroid on TSH release from the adenohypophysis, or the latter may result secondarily from further decrease in thyroid hormone production and/or alteration in the extra-thyroidal metabolism of iodide. The failure of cortisone to influence the I^{131} uptake of the thyroid depressed by PTU, does not preclude a direct effect of the steroid on the thyroid; uptakes are so diminished by PTU that further lowering may not be possible. The iodide-concentrating mechanism of the thyroid, however, does not appear to be involved(22).

Summary and conclusions. 1. Cortisone potentiates the effect of PTU on goitrogenesis in rats; a) thyroid and pituitary enlargement is greater than with goitrogen alone, and b) the respective changes in TSH levels of blood and pituitary are augmented. 2. The depressed avidity of thyroid tissue for I^{131} (with PTU) is not influenced by cortisone. 3. The results suggest that the potentiating action of cortisone on goitrogenesis involves augmentation of TSH secretion from the adenohypophysis. The implications are briefly discussed.

The authors are grateful to Mrs. Ada Schafer and Miss Sylvia Joffe for technical assistance.

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Suppression of Normal Bactericidal Action of Rabbit Serum Following Whole Body X-Irradiation.* (20303)

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The observations that x-irradiation injury results in the development of bacteremia(1), that the x-irradiated animal is more susceptible to infection(2), and that antibiotic therapy affords partial protection following mid-lethal to lethal exposure to x-irradiation(3) emphasize the significance of infection among the pathological processes which follow irradiation. Increase in susceptibility to infection and increased incidence of bacteremia are primarily due to the deleterious action of x-irradiation on the host defense mechanisms. Among the suppressed host defense mechanisms which have been implicated are: depressed antibody formation(4), depressed phagocytosis, and destruction of the normal mechanical fascial barriers against infection(5). Even with depression of the previously mentioned host defenses however one would expect neither the large incidence nor the degree of bacteremia encountered if the sera of x-irradiated animals maintained normal bactericidal activity. This latter function has been studied in rabbits; the present report is concerned with the suppression of the normal bactericidal action of rabbit serum following acute whole body x-irradiation.

Materials and methods. Albino rabbits weighing approximately 3 kg were irradiated in air by means of a Westinghouse Quadrocondex x-ray machine. Radiation factors were: 250 KV; 15 ma; 1.0 mm Al and 0.5 mm Cu filters in addition to an inherent filtration of 2.5 mm Al and 0.25 mm Cu; distance from focal point to surface on which rabbits were standing was 105 cms; the dose rate was approximately 25r per minute as measured by a Victoreen r meter. The whole body x-irradiation doses in these experiments varied from 500 to 725r with a 30-day mortality of 675 ± 11.3r as calculated by the method of Miller and Tainter(6). Group III (Table I) received 2 doses of 650r whole body x-irradiation with a 28-day interval between exposures. Blood was withdrawn by cardiac puncture or via the marginal ear vein from an equal number of x-irradiated and normal control rabbits at various intervals. The serum was separated, stored at 4°C and the serum bactericidal test performed within 30 hours. Bactericidal activity was tested in the following manner: 0.2 ml of blood serum was added to 1.8 ml of 0.9% NaCl solution (saline) and 0.25 ml of 1:100 dilution of an 18-hour nutrient broth culture of *Bacillus subtilis* (PCI 220) was added to the 1:10 dilution of serum. The serum, saline, culture suspension was placed in a 37°C water bath for 2 hours before the nutrient agar pour plates were prepared from various dilutions of the suspension. The plates were then incubated at 37°C for 48 hours and colony counts were made. Because of variation in the number of viable bacteria in the original inoculum it was neces-

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TABLE I. Effect of X-Irradiation Dose and Post-Irradiation Time on the Bactericidal Activity of Rabbit Serum.

Group No.	X-ray dose, r	Days after x-ray	Surviving rabbits	No. of sera with bactericidal activity	
				Normal	Depressed*
I	500	1	2	2	0
		7	2	2	0
		12	2	2	0
		16	2	2	0
II	650	1	6	6	0
		7	6	0	6
		13	6	0	6
		19	6	6	0
III	”	1	6	6	0
		5	6	3	3
		9	6	0	6
		15	6	0	6
		20	6	6	0
IIIA†	”	1	6	3	3
		5	6	0	6
		10	6	0	6
		15	3	0	3
		21	2	0	2
		25	2	0	2
		31	2	2	0
IV	”	1	6	6	0
		7	2	0	2
V	700	1	5	3	2
		7	5	2	3
		14	5	1	4
		19	5	5	0
VI	725	1	6	6	0
		5	6	2	4
		11	4	0	4

* Serum was considered to exhibit depressed bactericidal activity when the No. of viable organisms, following bactericidal test, was more than 3 times control group avg.

† Same animals as used in Group III exposed to 650 r 28 days after first x-irradiation exposure; total for this group 1300 r.

sary to test a control group of sera from normal rabbits at the same time as the x-irradiated group was being tested. As a check for bacteremia, 1.0 ml of whole blood was spread on a nutrient agar plate at the time blood was withdrawn for bactericidal testing. Whenever bacteremia was observed the same procedure used in the bactericidal test was carried out with the contaminated serum except that sterile saline was added in place of the culture dilution. With this technic the number of organisms of bacteremic origin was obtained. This number was subtracted from the number obtained following incubation of the test serum, saline, *B. subtilis* suspension to yield the number of viable organisms derived from the *B. subtilis* inoculum. In later experiments this control was abandoned since organisms of bacteremic origin were numerically insignificant by comparison with the test inoculum.

Results. With the technic described for measuring bactericidal activity of sera no difference was observed between sera from non-irradiated control and x-irradiated rabbits following exposure to 500r of whole body irradiation (Table I). After a single exposure to 650r or more of whole body x-irradiation, a loss of the bactericidal component was observed in all but one of the rabbits which lived 5 days or longer. Following the initial whole body x-irradiation the bactericidal activity of the sera of 2 rabbits was depressed on the first day following x-irradiation and the sera from the other 29 rabbits exhibited normal bactericidal activity at this time. When animals were exposed to a second 650r dose 28 days following the first 650r exposure, it was noted

TABLE II. Bactericidal Action of the Serum from X-Irradiated and Control Rabbits.

Treatment	Serum* dilution	Rabbit No.	Avg bacteria/ml†	Group avg
650 r	1:10	B–60	15200	12760
		61	16850	
		62	16500	
		63	9580	
		64	262	
		65	18160	
"	undil.	60	16630	6760
		61	3430	
		62	1450	
		63	148	
		64	726	
		65	2244	
0	1:10	C–60	6	6
		61	5	
		62	4	
		63	11	
		64	7	
		65	5	

* Serum withdrawn 9 days after a single 650 r x-irradiation exposure.

† Prior to 2 hr incubation period, each tube contained approximately 4840 bacteria/ml.

that the sera of 3 of the 6 animals in the group (Group IIIA) lost bactericidal activity within 24 hours whereas the sera of all 6 of these rabbits had exhibited normal bactericidal activity one day after initial x-irradiation exposure. The sera from all animals that lived 20 days following initial x-irradiation with either 650 or 700 r regained normal bactericidal action. The sera from the 2 rabbits which survived the second 650r dose had not regained normal bactericidal activity 25 days after the second exposure. This function was found to be normal on the thirty-first day.

It was frequently noted that the bactericidal action of sera from x-irradiated animals was depressed to the extent that multiplication of the bacteria took place during the 2-hour incubation period. This is shown in Table II and indicates that the bactericidal function of the sera was lost and that the organisms were multiplying during the incubation period. The second table also illustrates the differences observed between the bactericidal action of the sera from x-irradiated and normal rabbits and demonstrates that the bactericidal action of sera was quantitatively suppressed. It can be seen from Table II that a 1:10 dilution of serum from an x-irradiated rabbit may allow multiplication of bacteria during the incubation period while undiluted serum retains definite bactericidal action. The results show that undiluted sera from this group of x-irradiated rabbits were not as bactericidal as 1:10 dilutions of normal rabbit sera. It has been noted that with the described method for testing bactericidal action, counts of less than one organism per ml were obtained with undiluted normal rabbit sera.

Discussion. The results demonstrate that acute whole body x-irradiation in doses of 650r or more cause a depression in the bactericidal action of rabbit serum. It is possible that this loss or depression of serum bactericidal activity plays a major role in the increased susceptibility to infection which follows irradiation injury. The suppressed bactericidal activity following x-irradiation may be as detrimental to the host as the x-irradiation effects on other host defense mechanisms. Although the number of circulating phagocytes is reduced to about 10% of normal, it has been shown that whole body x-irradiation (350r) in the mouse does not alter the uptake of ThO_2 by the fixed phagocytes of the spleen and liver and that this treatment results in an increase in the per cent of peritoneal phagocytes containing engulfed bacteria following an intraperitoneal injection of *Micrococcus pyogenes* var. *aureus*(7).

The mechanisms involved in the loss of bactericidal activity from the sera of irradiated rabbits are as yet unknown but preliminary evidence indicates that the normal antibody or complement concentrations are not significantly altered following x-irradiation and a substance that inhibits the normal bactericidal component can not be demonstrated in the sera of x-irradiated rabbits. Buchner's (8) theory that leucocytes secrete the bactericidal substance found in normal serum could account for the loss of bactericidal action of serum following irradiation injury.

Summary. 1. Whole body x-irradiation of 650 to 725r results in depression of the normal bactericidal activity of rabbit serum. 2. With the x-irradiation doses employed, the bactericidal action of serum from rabbits is normal on the first post-irradiation day, is depressed by the fifth day following whole body

x-irradiation and returns in surviving animals by the twentieth post-irradiation day. 3. Following a second dose of 650r, serum bactericidal activity is depressed earlier and remains depressed for a longer time than after the first 650r x-irradiation exposure.

We wish to thank Mr. Garth Westenskow for carrying out the irradiation procedures in the Laboratory of Radiobiology, University of Utah, College of Medicine.

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Succinylmonocholine Iodide: Its Enzymatic Hydrolysis and Neuromuscular Activity.* (20304)

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In the past two years, succinylcholine has been used with increasing frequency for the production of muscular relaxation in the anesthetized patient. It was shown by Glick(1), Bovet-Nitti(2), and Castillo and de Beer(3), that succinylcholine is hydrolyzed by the plasma cholinesterase of various mammals. It was suggested by Ginzel *et al.*(4), by Whittaker and Wijesundera(5), and recently demonstrated by the latter(6) that the hydrolysis of succinylcholine by concentrated horse serum cholinesterase occurs in two steps. First, succinylcholine is hydrolyzed to succinylmonocholine and choline; succinylmonocholine is then very slowly hydrolyzed to succinic acid and choline. Because of its more rapid enzymatic hydrolysis in the human plasma(7), the mg/kg dose of succinylcholine necessary for the production of neuromuscular blockade, and the mg/kg/min dose used for the maintenance of muscular relaxation in anesthetized man is considerably greater than in other mammals(8).

Since under these circumstances the possibility of the accumulation of succinylmonocholine in anesthetized patients cannot be excluded, it seemed worthwhile to investigate the hydrolysis of succinylmonocholine in human plasma and to reinvestigate its neuromuscular effect in laboratory animals.

Methods. The hydrolysis of succinylmonocholine iodide[†] and succinyldicholine dichloride by normal heparinized and heat-inactivated heparinized human plasma was measured with Warburg's manometric technic at 37°C and pH 7.4 using a bicarbonate-carbonate buffer system. 0.4 ml of plasma or heat-inactivated plasma diluted to 1.6 ml with 0.025 M $NaHCO_3$ was placed in the main chamber of the Warburg vessels and 2 mg of succinyldicholine dichloride or succinylmonocholine iodide dissolved in 0.2 ml and titrated to pH 7.4 with NaOH was placed in the sidearms of the vessels. After the systems were brought into equilibrium with a 5% CO_2-95% O_2 gas mixture, the substrates were added to

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[†] The succinylmonocholine iodide used was supplied through the courtesy of Dr. E. J. de Beer of the Wellcome Research Laboratories, Tuckahoe, N. Y.

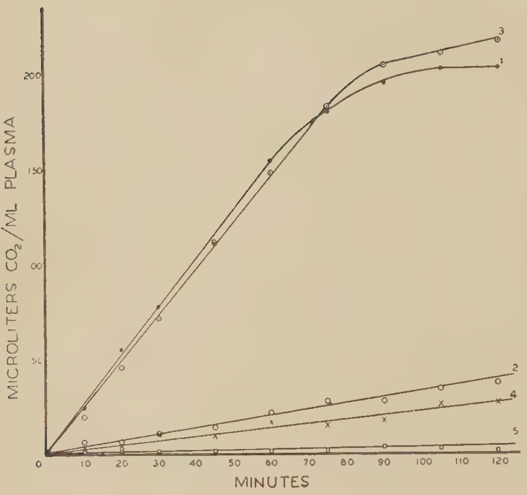

FIG. 1. Curve 1: Enzymatic hydrolysis of succinyldicholine dichloride. Curve 2: Enzymatic hydrolysis of succinylmonocholine iodide. Curve 3: Enzymatic hydrolysis of succinyldicholine dichloride and succinylmonocholine iodide. Curve 4: Nonenzymatic hydrolysis of succinyldicholine dichloride. Curve 5: Non-enzymatic hydrolysis of succinylmonocholine iodide. Substrate concentration 5.0 mg of succinyldicholine dichloride or succinylmonocholine iodide, or both (Curve 3), per ml of plasma.

the diluted plasma and the evolved CO_2 was measured at 10 minute intervals for 30 minutes and at 15 minute intervals thereafter for another 90 minutes. The results of the hydrolysis studies are presented in Fig. 1. The curves represent the average values obtained in duplicate observations with three different human plasmas. The curves of Fig. 1 indicate that the enzymatic hydrolysis rate of succinyldicholine dichloride (Curve 1) is about 8 times greater than that of succinylmonocholine iodide (Curve 2). The carbon dioxide produced by the enzymatic hydrolysis of mixtures of succinyldicholine dichloride and succinylmonocholine iodide (Curve 3), in the first 75 minutes was the same as that of the succinyldicholine component alone. Only after the hydrolysis of the dicholine component slowed down, did the quantity of carbon dioxide produced by the mixture surpass that produced by the dicholine component. The hydrolysis rate of succinyldicholine dichloride in heat-inactivated plasma (Curve 4) was considerable, but that of the succinylmonocholine iodide (Curve 5) was negligible.

The effect of succinylmonocholine iodide on neuromuscular transmission was observed on sciatic-gastrocnemius preparations of cats anesthetized with pentobarbital.‡ The result of a representative experiment is shown in Fig. 2. One mg per kg of succinylmonocholine iodide produced a definite neuromuscular block. The same dose repeated at 20-minute intervals showed a marked cumulative effect. On another animal, a 2.5 mg per kg dose produced complete neuromuscular block of 16 minutes duration and a partial block of about 40 minutes duration. In yet another cat, a 1 mg per kg dose of succinylmonocholine iodide administered 30 minutes after an 0.1 mg per kg dose of succinyldicholine dichloride caused a more profound neuromuscular block.

Comment. Our findings indicate that succinylmonocholine iodide is hydrolyzed by human plasma cholinesterase. The hydrolysis rate of succinylmonocholine iodide is about 8 times slower than that of succinyldicholine dichloride. The alkaline hydrolysis of succinylmonocholine iodide in the two-hour observation period was negligible.

In contrast to the observations of Bovet on rabbits(9) and to the statements made by Whittaker and Wijesundera(6), and Mayrhofer(10), succinylmonocholine iodide proved to be an effective inhibitor of neuromuscular transmission in cats. The onset of the neuromuscular blockade is somewhat slower and its duration is considerably longer than that of succinyldicholine dichloride and given at 20-minute intervals it showed marked cumulative effect. This fact together with the finding that a previous dose of succinyldicholine dichloride seems to have an additive effect on the action of succinylmonocholine iodide suggests that this compound might have a decamethionium-like effect on neuromuscular conduction.

Summary. The hydrolysis of succinylmonocholine iodide in normal and heat inactivated human plasmas was compared to the hydrolysis of succinyldicholine dichloride. In normal plasma, the hydrolysis rate of succinylmono-

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‡ The animal experiments were carried out in conjunction with Dr. C. H. Ellis and Mr. A. L. Wnuck of the Wellcome Research Laboratories, Tuckahoe, N. Y.

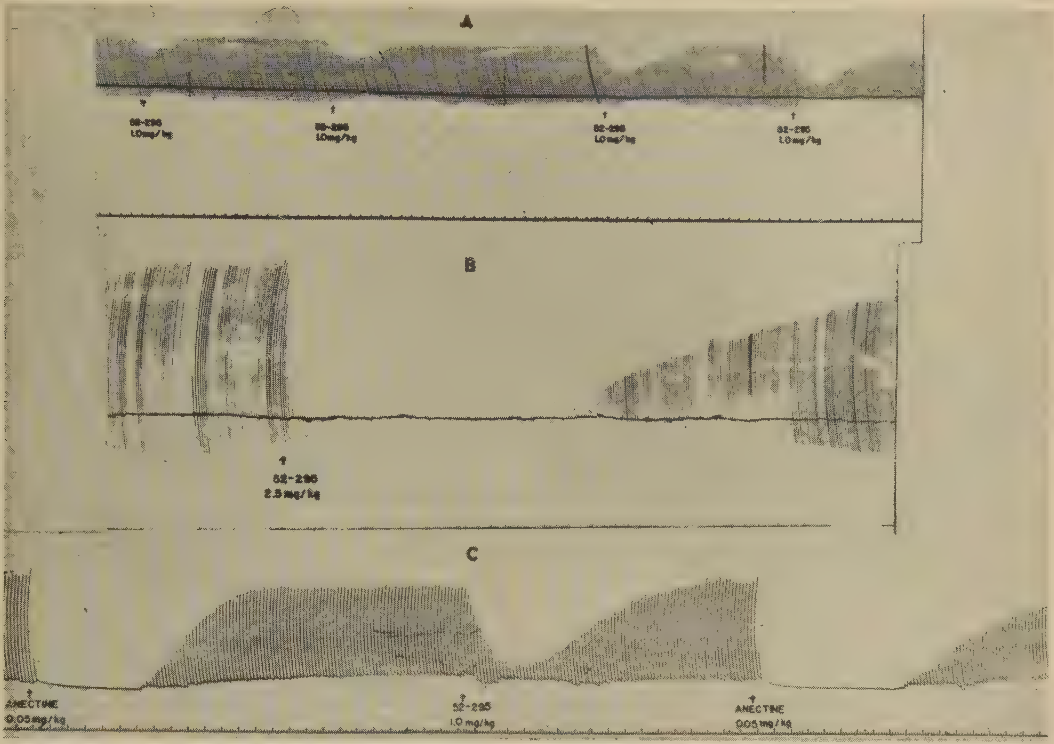

FIG. 2. A. The cumulative neuromuscular blocking action of 1 mg/kg succinylmonocholine iodide given at 20 min. intervals. B. The effect of a single 2.5 mg/kg dose. C. The mutually additive effects of succinyldicholine dichloride and succinylmonocholine iodide. Supramaximal stimuli with square wave pulses of 3 milliseconds duration at 6 sec. intervals. Time signal: 1 min.

choline iodide was found to be about 8 times slower than that of succinyldicholine dichloride. In heat inactivated plasma, the hydrolysis rate of succinylmonocholine iodide was negligible in the 2-hour observation period. Succinylmonocholine iodide in 1.0 to 2.5 mg/kg doses inhibited neuromuscular transmission in the sciatic-gastrocnemius preparation of cats.

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Initial Stages of Mustard Action Upon Influenzal Toxicity and Infectivity.*

(20305)

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A previous paper(1) has shown that exposure of influenza A (PR8) virus to appropriate concentrations of sulfur mustard or various chloroethylamine derivatives resulted in considerable inactivation of the toxic and infective principles of virus. Analysis of the time relationship in inactivation of these two viral attributes demonstrated the rapidity of destruction of both viral properties and a seemingly greater inactivation of viral infectivity as opposed to viral toxicity. In this earlier investigation it had not been possible to study the initial phases of mustard action upon influenzal toxicity because of the complicating factor of chemical intoxication following intracerebral inoculation of materials containing unreacted mustard or its intermediates. The possibility of either an equivalent rate of destruction of viral toxicity and infectivity or a selective action of mustard upon one or the other of the two viral characteristics in the initial stages of mustard-virus interaction was, therefore, not ascertained. The present report describes the use of sodium thiosulfate as a means of circumventing chemical intoxication and the nature of mustard action upon influenzal toxicity and infectivity in the early periods following exposure of influenza A (PR8) virus to one of the chloroethylamine derivatives.

Materials and methods. Virus. An egg-adapted strain of influenza A (PR8) virus with a continuous history of allantoic passage in embryonated eggs since isolation was used in these studies. Virus was prepared by suballantoic inoculation of 10- or 11-day embryonated eggs with 0.05 ml of a 10^{-6} or 10^{-7} dilution of virus in beef-heart infusion broth. After incubation of infected eggs at 36°C for 40 to 48 hours, the eggs were chilled overnight in the refrigerator and the allantoic fluids harvested. The pooled fluids were stored in the refrigerator and centrifuged at 2400 rpm for 20 minutes before use to remove coarse particles which had accumulated upon standing. The toxicity and infectivity of the preparation was determined each week, and whenever any significant loss of activity in either of the viral properties was noted, a new suspension of infected allantoic fluid was prepared. *Mustard.* The chloroethylamine derivative used throughout these investigations was cyclohexyl [bis(β-chloroethyl)] amine hydrochloride, kindly supplied by Dr. H. B. Woodruff of Merck & Co., Inc. A 0.05 M solution of chemical was prepared by dissolving weighed quantities of the chemical in cold, triple-distilled water of pH 2.6. This was done to eliminate variations in ionic content of water and to repress cyclization of mustard until ready for addition to virus. *Exposure of virus to mustard.* An appropriate amount of the 0.05 M solution of mustard was added to a given volume of chilled viral suspension to yield the desired concentration of chemical. The mustard-virus suspension was thoroughly mixed and placed in an ice-water bath at 4°C. Samples were removed at selected intervals for determination of viral toxicity and infectivity. Only freshly prepared solutions of mustard were employed in these tests and they were added to virus as soon as complete dissolution of chemical had occurred. *Concentration of mustard-treated virus.* Suspensions of mustard-treated virus were adsorbed with 5% chicken red cells in the cold. After 45 minutes in an ice-water bath, the supernatant fluid was removed as completely as possible, and the agglutinated cells washed with 0.1 M phosphate buffer. The washed cells were allowed to resettle in the cold. Following removal of the supernatant fluid, a volume of 0.1 M phosphate buffer of pH 7.4, equivalent to 1/10th of the original volume of mustard-virus suspension, was added to the agglutinated cells. Elution of adsorbed virus was then carried out in a 37°C water bath for

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* This work was aided by a grant from the Research Committee of the University of California.

4 hours. At the end of this period the suspension was centrifuged at 2400 rpm for 20 minutes and the supernatant fluid removed and tested for viral toxicity, infectivity and hemagglutinins. *Test of viral toxicity.* At the time selected for testing of mustard-treated virus for toxicity, 0.04 ml of a 5 M aqueous solution of sodium thiosulfate was mixed with 1.96 ml of mustard-virus suspension. This represented the undiluted mustard-virus preparation from which serial 2-fold dilutions of virus were made in 0.1 M aqueous sodium thiosulfate for determination of residual toxicity of virus. The test for viral toxicity was made by inoculating 0.03 ml of virus into the right cerebral hemisphere of mice under light ether anesthesia. The animals used in the tests were Swiss mice (Webster strain), 12 to 15 g weight and of random sex. Mice which were obviously traumatized by the intracerebral injections were discarded. Deaths or convulsions (upon spinning of the animals) 24 hours after injection were used as criteria for virus-induced toxic reactions. All mice were observed daily for a period of 7 days. The dilution of virus causing toxic reactions in 50% of the experimental animals was calculated by the method of Reed and Muench(2). *Toxicity of viral progeny derived from mustard-treated virus.* The toxicity of viral progeny derived from mustard-treated virus after one allantoic passage in 10- or 11-day embryonated eggs was determined in a similar manner. The propagation of mustard-treated virus was carried out in the manner described below for the infectivity test. Only the fluids from eggs infected with a single infective dose of mustard-treated virus were tested in this respect. *Infectivity test.* Mustard-treated virus was diluted serially in 10-fold steps in beef-heart infusion broth. The starting point of the dilution series was the undiluted sample of mustard-virus suspension containing 0.1 M sodium thiosulfate as prepared for determination of viral toxicity. The dilutions of virus were inoculated suballantoically in 0.05 ml amounts into groups of 4 to 6 embryonated eggs of 10- or 11-days incubation. After 40 to 48 hours at 36°C, the individual allantoic fluids were tested for presence of viral hemagglutinins by mixing 0.1 ml of fluid with 0.25 ml of 1% chicken erythrocytes. The test was read by the pattern method(3) after 30 minutes at room temperature. The ID_{50} per 0.05 ml of suspension was calculated by the method of Reed and Muench(2). *Hemagglutination test.* Titration of viral hemagglutinins in the mustard-virus suspensions concentrated by the method of red cell adsorption and elution was made by using serial 2-fold dilutions of virus in saline and mixing 0.5 ml of viral dilution with 0.5 ml of 1% chicken red cells. The test was read after 30 minutes at room temperature by the pattern method(3). The end point was taken as the last tube showing complete agglutination, and the titer expressed as the reciprocal of the dilution of virus in this tube.

Results. Effect of sodium thiosulfate upon viral toxicity and infectivity. Studies of the action of aqueous solutions of sodium thiosulfate upon the toxic and infective components of influenza A (PR8) virus (results not shown) revealed that concentrations of 0.2 M thiosulfate or less caused no inactivation of either of the viral attributes. The dilution of virus causing toxic reactions in 50% of experimental animals and the number of egg-infective doses remaining after contact of virus with 0.2 M sodium thiosulfate for one hour at room temperature (24°C) were identical with the values observed for untreated virus. The action of higher concentrations of sodium thiosulfate upon virus was not investigated.

Toxicity of sodium thiosulfate for intracerebrally injected mice. The tolerance of mice for intracerebral inoculation of 0.03 ml of various concentrations of aqueous solutions of sodium thiosulfate is shown in Table I. The data of this table represent the combined results of two separate experiments. It is evident that concentrations of 0.075 M or less of sodium thiosulfate were well tolerated. No fatalities or ill effects were observed in any of the animals during an observation period of 7 days. Intracerebral injections of 0.1 M sodium thiosulfate proved toxic to slightly less than 50% of experimental animals. Still higher concentrations of chemical (0.25 to 0.5 M) were 100% lethal.

Reduction of mustard toxicity by sodium

TABLE I. Toxicity of Sodium Thiosulfate for Intracerebrally Injected Mice.

	(M) sodium thiosulfate inoculated						
	.5	.25	.125	.1	.075	.065	.05
No. dead / No. inoc.	16/16	16/16	8/16	7/16	0/16	0/16	0/16

TABLE II. Reduction of Mustard Toxicity by Sodium Thiosulfate.

(M) mustard in allantoic fluid	(M) thiosulfate in allantoic fluid	Intracerebral toxicity of sample Min. after dissolution of mustard			
		0	5	15	30
5×10^{-4}	0	6/6*	6/6	6/6	4/6
"	.075	0/6	0/6	0/6	0/6
"	.1	0/6	—	—	0/6
0	.075	0/6			
"	.1	2/6			

* Numerator designates No. of animals dead. Denominator indicates total No. of animals in test.

— indicates sample not tested.

TABLE III. Initial Stages of Mustard Action upon Influenzal Toxicity and Infectivity.

(M) mustard added to virus	Time (min.) of sampling	Toxicity of sample				Dilution causing 50% toxic reaction of sample	Log ID_{50}
		Undil.	1:2	1:4	1:8		
5×10^{-4}	Immediate	3/6*	1/6	0/6	0/6	1:1.1	7.5
"	5	1/6	0/6	—	—	<1:1	7.0
"	10	1/6	0/6	—	—	"	6.3
"	20	0/6	—	—	—	"	5.5
"	30	0/6	—	—	—	"	5.2
0		6/6	5/6	4/6	0/6	1:4.3	7.5

* Numerator refers to No. of animals showing toxic symptoms. Denominator refers to total No. of animals in test.

— indicates sample not tested.

thiosulfate. It may be seen from the results of Table II that intracerebral inoculation of 0.03 ml of 5 x 10^{-4} M mustard in normal allantoic fluid proved highly toxic to mice. All of the animals succumbed within a few hours after inoculation. The toxicity of the preparation was almost as great after 30 minutes at room temperature as in the first few minutes. Addition of 0.075 M sodium thiosulfate to the mustard-containing allantoic fluid immediately eliminated the lethality of the preparation, for none of the experimental animals given mustard-thiosulfate allantoic fluid died during the observation period of 7 days. It is of interest that admixture of 0.1 M sodium thiosulfate with mustard-containing allantoic fluid not only rendered the latter entirely innocuous to mice, but resulted also in reduction of the toxicity of sodium thiosulfate itself. The nullifying action of sodium thiosulfate upon mustard toxicity thus provided a means for studying the toxicity of virus during the early stages of mustard-virus interaction without the complicating factor of chemical intoxication.

Nature of mustard action upon influenzal toxicity and infectivity in the early stages of mustard-virus interaction. The data of the experiment shown in Table III are representative of the results obtained in several identical experiments. There seemed to be an extremely rapid destruction of the toxic component(s) of virus following addition of mustard to virus. As compared with the toxic content of untreated virus, and reflected in the dilution of virus causing toxic reactions in 50% of animals, an approximate 4-fold drop in viral toxicity may be observed for mustard-treated virus within the first few moments of reaction. In the succeeding intervals (5 to 30 minutes) intracerebral injection of mice with mustard-virus suspension provoked only an occasional

TABLE IV. Toxicity of Mustard-Treated Virus after Concentration by Red Cell Adsorption and Elution.

Sample[*]	(M) mustard added to virus	HA[†] titer	Toxicity of sample Undil.	1:2	1:4	1:8	Dilution causing 50% toxic reaction
MV	5×10^{-4}	512	0/6[‡]	—	—	—	<1:1
MVC	"	4096	0/6	0/6	0/6	—	"
MV	1.25×10^{-4}	512	6/6	3/6	0/6	—	1:2
MVC	"	4096	5/6	5/6	3/6	1/6	1:3.6
Virus	0	512	6/6	6/6	5/6	2/6	1:6.1

[*] MV refers to mustard-treated virus after 30 min. at 4°C. MVC refers to same preparation after concentration.

[†] Hemagglutinin titer expressed as reciprocal of dilution of virus in last tube showing complete agglutination.

[‡] No. of animals showing toxic symptoms/No. of mice in test.

toxic response. In contrast to the findings recorded for the action of mustard upon viral toxicity, there was no immediately observable inactivation of the infective component(s) of influenza virus by mustard. Instead, the course of inactivation of viral infectivity appeared to be a gradual and orderly process, as evidenced by the progressive reduction in level of egg-infective virus at each of the succeeding intervals.

Toxicity of mustard-treated virus after concentration by red cell adsorption and elution. The results shown in Table III have established the rapidity of destruction of influenzal toxicity by 5 x 10^{-4} M mustard, but carry no inferences concerning the completeness or permanency of this loss. These two aspects were investigated by a) analysis of the toxicity of mustard-treated virus after concentration of virus by the method of red cell adsorption and elution, and b) study of viral progeny obtained from eggs infected with one ID$_{50}$ of mustard-treated virus. The results are presented in this and the following section.

Table IV indicates that destruction of influenzal toxicity by 5 x 10^{-4} M mustard was apparently complete after 30 minutes at 4°C. This was suggested by the absence of toxic reactions in mice given intracerebral injections of mustard-treated virus which had been concentrated 8-fold (as reflected in an increased hemagglutinating titer). In comparison, it may be seen that exposure of virus to 1.25 x 10^{-4} M mustard for the same period of time resulted in only an approximate 3-fold drop in viral toxicity. An 8-fold concentration of this particular preparation produced almost a doubling of the residual viral toxicity. That the increased toxicity of the latter was not ascribable to chemical intoxication was indicated by the absence of toxicity of the concentrate prepared from the viral suspension treated with 5 x 10^{-4} M mustard.

Toxicity of viral progeny derived from mustard-treated virus. Table V shows the toxic behavior of a few selected examples of viral progeny derived from mustard-treated virus. It is readily evident that viral progeny of high and low toxicity may be encountered and that no definite relationship between hemagglutinin titer and level of viral toxicity can be detected. This particular behavior of virus derived from cultivation of single infective units of mustard-treated virus in embryonated eggs closely simulated the toxic pattern of untreated virus passaged in an identical manner (results for latter not shown). Examination of over twenty such single viral isolates revealed no instances of non-toxic viral suspensions.

Discussion. Previous investigations of the action of mustards upon viruses have been confined largely to problems of pragmatic significance, and utilization of these chemi-

TABLE V. Toxicity of Viral Progeny Derived from Mustard-Treated Virus.

Sample	HA titer	Toxicity of sample Undil.	1:2	1:4	Dilution causing 50% toxic reaction
1	320	6/6[*]	6/6	4/6	>1:4
2	"	"	3/6	0/6	1:2
3	"	5/6	0/6	"	1:1.3

[*] No. of animals showing toxic symptoms/No. of mice in test.

cals as selective agents for inactivation of functional groups of viruses has not been explored in general. An earlier report(1) on the action of mustards upon influenzal toxicity and infectivity had demonstrated a concurrent and rapid inactivation of both viral attributes and a seeming lack of preferential action of mustards upon virus. These findings, however, represented essentially the end results of mustard action, and the possibility of an early preferential action of mustard upon virus was not excluded. Obvious hindrances to an analysis of the initial stages of mustard action upon influenzal toxicity and infectivity were extreme rapidity of mustard action and residual chemical toxicity. As described in another paper(4), deceleration of mustard inactivation of virus was attained by a reduction in temperature of the reacting system. The present report demonstrated the detoxification of residual mustard toxicity by sodium thiosulfate. Utilization of these two observations in studying the early phases of mustard-virus interaction has shown a seemingly rapid destruction of viral toxicity and a more gradual but progressive inactivation of viral infectivity by cyclo mustard. The results were suggestive of an early preferential action of mustard upon influenza A (PR8) virus. While the possibility of a continuing action of mustard upon virus following inoculation of mustard-virus suspension into the central nervous system cannot be eliminated, it does not seem too likely in view of the dilution factor and the many reactive constituents in brain tissue.

Various studies(5-8) have shown that mustards increase mutation rates in *Drosophila*, bacteria and fungi. It might be assumed that viruses may likewise be subject to the mutational effect of these chemicals. The findings reported herein showed that, despite the apparently complete destruction of viral toxicity as evidenced by inability to restore this property after concentration of mustard-treated virus, no lasting effects were demonstrable. This was indicated by the occurrence of toxic manifestations in animals receiving intracerebral injections of viral progeny derived from mustard-treated virus.

Summary. 1. Sodium thiosulfate in concentrations of 0.2 M or less produced no observable effects upon viral toxicity and infectivity. 2. Intracerebral inoculation of 0.075 M or less of sodium thiosulfate was well tolerated by mice. 3. Admixture of 0.1 M sodium thiosulfate with 5 x 10^{-4} M cyclo mustard resulted in detoxification of the latter. 4. A possibly greater rate of destruction of viral toxicity by mustard, as opposed to viral infectivity, seemed to occur in the early stages of mustard-virus interaction. 5. Inactivation of viral toxicity by mustard proved complete, but the loss was not demonstrable in the viral progeny derived from eggs infected with one ID_{50} of mustard-treated virus.

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